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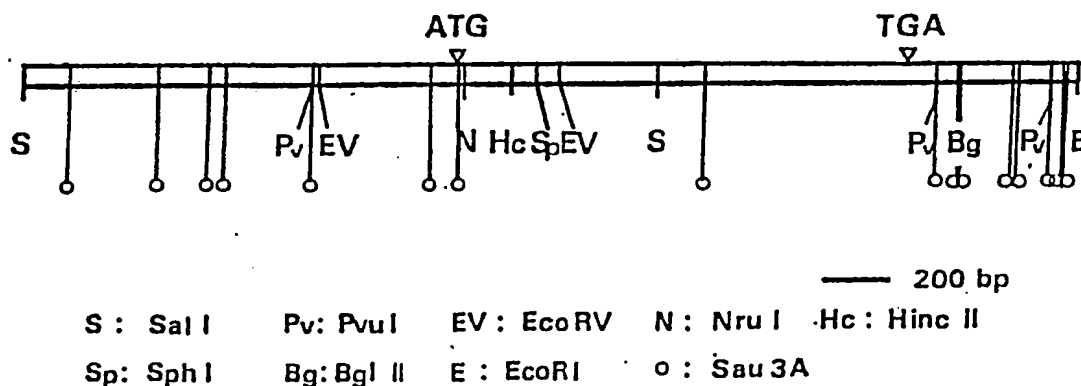
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(54) Title: NOVEL ENZYME



Restriction map of SSE fragment (3.1 kb)

ATG: initiation codon TGA: stop codon

(57) Abstract

A novel coenzyme independent L-sorbose dehydrogenase originating from a microorganism belonging to the genus *Gluconobacter oxydans* which acts on L-sorbose to produce 2-keto-L-gulonic acid. The enzyme has the following physico-chemical properties: a) optimum pH: about 7.0, b) optimum temperature: about 30°C to about 40°C, c) molecular structure: consisting of one type of unit having a molecular weight of about $47,500 \pm 5,000$ as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis, d) thermostability: stable below 30°C, and e) inhibition: by Cu^{2+} ions.

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Novel enzyme

10 The present invention relates to a novel enzyme, namely
L-sorbose dehydrogenase, a process for producing the same
and a process for producing 2-keto-L-gulonic acid utilizing
said enzyme. Moreover, the present invention relates to
genetic engineering techniques which provide an improved
15 method for the cloning and expression of the gene of said
enzyme and transformed microorganisms capable of producing
2-keto-L-gulonic acid with high efficiency.

The compound 2-keto-L-gulonic acid (2-KGA) is an
20 important intermediate in the synthesis of ascorbic acid
(Vitamin C). Numerous microorganisms are known to produce
2-KGA from D-sorbitol or L-sorbose, for example, members of
the genera Acetobacter and Pseudomonas, though levels of
2-KGA produced are less than 6g/L (Japan Patent Publication
25 No. 40,154/1976). Generally the pathway of D-sorbitol to
2-KGA can be illustrated as follows (Makover et al. 1975,
Biotechnol. and Bioeng. 17, 1485-1514):



30

Reactions to convert L-sorbose to 2-KGA using
microorganisms are known. 2-KGA production from L-sorbose
using cell free extracts of microorganisms was reported in
several prior publications.

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In US-PS No. 3,907,639, microorganisms belonging to the genera Acetobacter, Pseudomonas, Escherichia, Serratia, Bacillus, Staphylococcus, Aerobacter, Alcaligenes, Penicillium, Candida and Gluconobacter were reported to be
5 capable of such a conversion.

Furthermore, Kitamura et al. (Europ. J. Appl. Microbiol., (2) 1, 1975) reported that a L-sorbose oxidizing enzyme found in Gluconobacter melanogenes IFO 3293
10 required neither coenzyme nor an electron acceptor for the development of its enzyme activity.

However, no disclosure has been made up to now on a purified enzyme having the activity to oxidize L-sorbose
15 to 2-KGA not depending on coenzymes, e.g. nicotinamide-adenine dinucleotide (NAD) or nicotinamide-adenine dinucleotide phosphate (NADP), etc. It has been found that the purified enzyme isolated from the membrane fraction of
20 cells of specific microorganisms catalyzes the oxidation of L-sorbose to 2-KGA independently of coenzymes such as NAD and NADP. The present invention has been accomplished based on this finding.

It is an object of the present invention to provide a
25 novel coenzyme independent L-sorbose dehydrogenase which catalyzes oxidation of L-sorbose to 2-KGA. It is another object to provide a process for producing said novel L-sorbose dehydrogenase by a fermentation method. It is
also an object to provide an improved process for the
30 production of 2-KGA from L-sorbose with the aid of said novel L-sorbose dehydrogenase or a microorganism which produces said enzyme.

Furthermore, another aspect of the object of the present
35 invention is to provide gene engineering techniques which enable the production of said L-sorbose dehydrogenase by

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an improved method and also enable the production of 2-KGA from L-sorbose using said enzyme produced by a recombinant microorganism or using said recombinant microorganism by fermentation. In this respect, DNA comprising the gene
5 encoding said novel L-sorbose dehydrogenase, vectors and recombinant organisms containing said DNA are also within the scope of the present invention.

It is to be understood that with regard to the partial
10 amino acid sequences given in connection with the DNA, the DNA fragments, the recombinant DNA molecules, the recombinant microorganisms and the processes involved, the functional equivalents of said amino acid sequences, i.e. equivalents which achieve the same goal are also included.

15

Description of the Drawings

Figure 1 illustrates the restriction map of the plasmid pVK 102.

20

Figure 2 illustrates the restriction maps of the plasmids of the present invention.

Figure 3 illustrates the restriction map of a DNA
25 containing the structural gene encoding L-sorbose dehydrogenase.

Figure 4 illustrates the DNA sequence encoding L-sorbose dehydrogenase originated from *Gluconobacter*
30 oxydans IFO 12258.

The underlined portions indicate partial amino acid sequences also determined by amino acid sequence analysis of peptide fragments of the enzyme protein.

35

The novel L-sorbose dehydrogenase of the present invention is characteristic in its independency on coenzymes

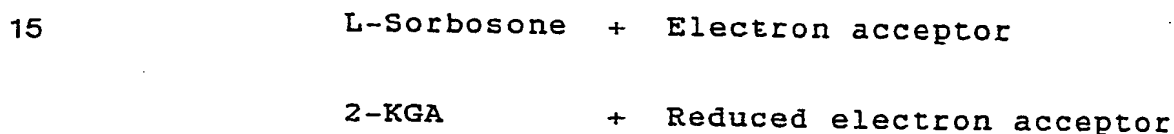
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such as NAD. NADP, flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD), etc. in the oxidizing reaction from L-sorbose to 2-KGA.

- 5 The properties of the enzyme and the production method may be summarized as follows:

(1) Enzyme activity

- 10 The L-sorbose dehydrogenase of the present invention catalyzes the oxidation of L-sorbose to 2-KGA in the presence of an electron acceptor according to the following reaction;



The enzyme does not utilize molecular oxygen as an
20 acceptor. As an acceptor, 2,6-dichlorophenolindophenol (DCIP), phenazine methosulphate, Wurster's blue, ferricyanide, coenzyme Q or cytochrome c can be used.

Enzyme assay was performed at 25°C by measuring the
25 decrease of absorbance at 600nm of DCIP spectrophotometrically. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the reduction of 1 μ mole of DCIP per minute.

- 30 The extinction coefficient of DCIP at pH 7.0 was taken as 9.45mM^{-1} . The basal reaction mixture is shown below. The mixture was prepared just before the assay.

Basal mixture:

- 35 0.1M Potassium phosphate
buffer (pH 7.0) containing
0.3% Triton X-100 6ml

- 5 -

2.5mM DCIP

0.45ml

H₂O

10.35ml

A cuvette with 1cm light path contained 0.4ml of basal
5 mixture, 20 μ l of 10mM phenazine methosulphate, 10 μ l of
enzyme solution and 20 μ l of 110mM L-sorbose solution in
a final volume of 0.45ml. A reference cuvette contains all
components except the substrate. The reaction was initiated
by the addition of the substrate. Enzyme activity was
10 measured as the initial reduction rate of DCIP.

(2) Substrate specificity

The substrate specificity of the enzyme can be
15 determined by the same enzyme assay method as described
above under (1) using various substrate solutions. The
enzyme of the present invention catalyzes the oxidation of
various types of aldehyde compounds as exemplified in
Example 3 shown later on.

20

(3) Physico-chemical properties

The enzyme of the present invention has the following
physico-chemical properties:

25

- a) Optimum pH: about 7.0,
- b) Optimum temperature about 30° to about 40°C,
- c) Molecular structure: the enzyme consists of one
single type of units having a molecular weight of
30 about 47,500 \pm 5,000 as measured by sodium dodecyl
sulfate polyacrylamide gel electrophoresis,
- d) Thermostability: stable below 30°C,
- e) Inhibitor: inhibited by Cu²⁺-ions.

35

It has to be noted that the optimum temperature range
relates to the temperature range where the enzyme exhibits
high initial reaction rates, independently of the fact that

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the enzyme may be decomposed after incubation of longer periods of time at the optimum temperature range.

(4) Production of the enzyme

5

The L-sorbose dehydrogenase provided by the present invention can be prepared by cultivating an appropriate microorganism, disrupting the cells and isolating and purifying it from the cell free extract of disrupted cells, 10 preferably from the membrane fraction of the microorganism.

The microorganisms used for the present invention are microorganisms belonging to genus *Gluconobacter* or mutants thereof. According to the newest classification, all the 15 strains belonging to *Gluconobacter* fall into the species *Gluconobacter oxydans*.

Morphological and physiological characteristics of the strains belonging to *Gluconobacter oxydans* are described in 20 "Bergey's Manual of Systematic Bacteriology", Vol. I, p. 275-278, 1984 and F. Gosselle et al., International J. System. Bacteriol. Vol. 33, p. 65-81, 1983.

Microorganisms belonging to the genus *Gluconobacter* 25 which are used in the present invention can be isolated from natural sources or are available from culture collections. The mutants derived thereof may also be used according to the present invention.

30 The mutants used in the present invention can be obtained by treating a wild type strain with a mutagen such as ultraviolet irradiation, X-ray irradiation, γ -ray irradiation or contact with nitrous acid or other suitable mutagens, or by isolating a clone occurring by spontaneous 35 mutation. These mutations of a wild type strain or a mutant strain thereof may be effected in any of the ways well known per se for this purpose by those skilled in the art. Many

of these methods have been described in various publications, see for example, "Chemical Mutagens" edited by Y. Tajima, T. Yoshida and T. Kada, published by Kodansha Scientific Inc., Tokyo, Japan, in 1973.

5

The mutants according to the present invention can also be obtained by fusion of the strains belonging to the species *Gluconobacter oxydans* and the combination of mutagenesis and/or fusion.

10

Examples of the strains most preferably used in the present invention are *Gluconobacter oxydans* (listed as melanogenes in the catalogues) IFO 12257, *Gluconobacter oxydans* (listed as melanogenes in the catalogues) IFO 12258
15 and the like.

The microorganisms may be cultured in an aqueous medium supplemented with appropriate nutrients under aerobic conditions. The cultivation may be conducted at pH of 4.0
20 to about 8.0, preferably from 5.5 to 7.5. The cultivation period varies depending upon the microorganisms and nutrient medium to be used, it is preferably about 10 to 100 hours. A preferred temperature range for carrying out for the cultivation is from about 10°C to 40°C, preferably from 25°C
25 to 30°C.

It is usually required that the culture medium contains nutrients such as assimilable carbon sources, e.g. glycerol, D-mannitol, D-sorbitol, erythritol, ribitol, xylitol,
30 arabitol, inositol, dulcitol, D-ribose, maltose and sucrose, preferably L-sorbose, D-sorbitol or glycerol; digestible nitrogen sources such as organic substances, for example, peptone, yeast extract, soybean meal and corn steep liquor, and inorganic substances, for example, ammonium sulfate,
35 ammonium chloride and potassium nitrite; vitamins and trace elements.

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Below, a summary of one of the embodiments for isolation and purification of L-sorbose dehydrogenase from the microorganisms after the cultivation is described.

- 5 (1) Cells are harvested from the fermentation broth by centrifugation.
- (2) The cells are suspended in the buffer solution and disrupted by means of a homogenizer, sonicator or
10 treatment with lysozyme and the like to give a disrupted solution of cells.
- (3) L-sorbose dehydrogenase is isolated and purified from the cell free extract of disrupted cells,
15 preferably from the membrane fraction of microorganisms.

The L-sorbose dehydrogenase provided by the present invention is useful as a catalyst for the production of
20 2-KGA from L-sorbose. The reaction should be conducted at pH values of from about 5.0 to about 10.0 in the presence of an electron acceptor, for example, DCIP, phenazine methosulfate, Wurster's blue, ferricyanide, coenzyme Q, cytochrome c and the like, in a solvent such as phosphate
25 buffer, tris-HCl buffer and the like. A preferred temperature range of carrying out the reaction is from about 10°C to about 50°C. When the pH and temperature are set at about 7.0-8.0 and 30°C, respectively, the reaction usually brings about most preferable results. The concentration of
30 L-sorbose in a solvent may vary depending on other reaction conditions, but, in general, it is desirable about 10-100g/L, most preferably about 30-40g/L.

In the reaction, the enzyme may also be used in an
35 immobilized state with an appropriate carrier. Any means of immobilizing enzymes generally known to the art may be used. For instance, the enzyme may be bound directly to a

membrane, granules or the like of a resin having functional group(s), or it may be bound to the resin through bridging compounds having bifunctional group(s), for example, glutaraldehyde.

5

Further to the enzyme as such, the process for producing the same and the process for producing 2-KGA using the enzyme outlined above, the present invention encompasses a genetic engineering technique for cloning and expression of the enzyme gene, genetic materials useful for the production of the enzyme, and an improved process for the production of 2-KGA using said genetic materials.

More particularly, the present invention relates to the production of coenzyme independent L-sorbose dehydrogenase using a recombinant microorganism having introduced a recombinant DNA molecule which contains a gene encoding a polypeptide having the activity of the enzyme, and said recombinant microorganism per se.

20

The present invention also relates to the gene and the recombinant molecule containing said gene which can be used to construct the above recombinant microorganism.

25

Furthermore, the present invention relates to a process for producing 2-KGA by contacting L-sorbose with the enzyme produced from said recombinant microorganism and processes for producing 2-KGA from D-sorbitol, L-sorbose or L-sorbose by fermentation with the above recombinant microorganism.

30

Finally, the present invention also relates to a process for producing a transconjugant of the above recombinant microorganism using Gluconobacter strains.

35

Briefly, the recombinant microorganism utilized in the present invention can be obtained by the following steps:

- 10 -

- (1) Constructing a genomic library from the chromosomal DNA of an appropriate strain belonging to the genus *Gluconobacter* which can produce coenzyme independent L-sorbose dehydrogenase,
- 5 (2) Screening the above genomic library to obtain a clone which expresses the enzyme or its derivative,
- (3) If desired, subcloning of the above clone to obtain a subclone which contains a smaller size of the DNA
10 fragment necessary for the expression of the enzyme, and
- (4) If desired, reconstruction of the recombinant DNA
15 molecule to enable high productivity.

The materials and the techniques used in the above aspect of the present invention are explained in details as follows:

20

Cloning of the gene encoding the novel L-sorbose dehydrogenase and the construction of a recombinant microorganism

25 A) Origin of the gene

The gene encoding coenzyme independent L-sorbose dehydrogenase of the present invention can be cloned from microorganisms belonging to the genus *Gluconobacter* which can produce said enzyme, preferably from
30 *Gluconobacter oxydans* IFO 12258, *Gluconobacter oxydans* IFO 12257 or a mutant thereof.

B) Construction of a genomic library

35

By a procedure well known in the art, the chromosomal DNA isolated from the above origin strain is partially

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digested with a restriction enzyme such as Sal I, Hind III, Xho I, or EcoR I. The resulting DNA fragments having large molecular size, preferably 15 to 35kb, are collected and ligated with a suitable vector DNA which is digested with an appropriate restriction enzyme and preferably treated with bacterial or calf intestine alkaline phosphatase. As the vector, either a plasmid or phage vehicle can be utilized. In the present invention, the most preferable vector is a cosmid vector, for example, pVK100 (ATCC 37156) or pVK102 (ATCC 37158), or a derivative thereof, i.e. a derivative harboring a cos site of the λ phase, Mob site, a replication origin of RK2 and one or more marker genes such as antibiotics resistance genes.

Thus, obtained recombinant DNAs on suitable vectors are introduced into a suitable host organism using any of DNA transfer system, for example, transformation, transduction or conjugal mating.

In the present case, the genomic library consisting of the above obtained recombinant DNAs can be suitably constructed in a strain of *Gluconobacter oxydans* as a host by the following procedure:

- (1) To obtain a genomic library in a strain of *Gluconobacter oxydans*, the recombinant DNAs are firstly transferred into any strain of *Escherichia coli* to construct a genomic library in *E. coli*. For this purpose, the in vitro packaging system of lamda phage wherein the vector is a cosmid vector can be used. This system using a cosmid vector is suitable for the cloning of a large size DNA. A preferable host for the in vitro packaging system is *E. coli* C600, *E. coli* HB101 or *E. coli* ED8767.
- The in vitro packaging system is commercially available and can be used according to the manufacturer's instructions. Such a system can be also prepared and

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used according to the description in the literature, e.g. T. Maniatis et al., Molecular Cloning, A Laboratory Manual, 256-268 (1982), Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

5

- (2) Then, the recombinant DNAs in a strain of E.coli as a donor are transferred into a suitable strain of Gluconobacter as a recipient using bi-parental conjugal mating or tri-parental conjugal mating.

10

When bi-parental conjugal mating is made use of, it is necessary that the donor strain of E.coli possesses Tra genes on its chromosomal DNA. And when the genomic library in E.coli is constructed in a strain not having Tra genes, the recombinant DNAs should be transferred into another E.coli strain having Tra genes, E. coli S17-1 can be preferably used as such a donor strain. The recombinant DNAs in one strain of E.coli can be transferred into the other strain having Tra genes by usual transformation method to construct a new genomic library in E. coli which can be used as a donor strain in conjugal mating.

15

20

It is also required for the bi-parental conjugal mating that the vehicle which should be transferred by conjugal mating possesses a mob site. The mentioned vectors pVK100, pVK102 or its derivatives having Mob site are also preferred to construct said vehicle having Mob site.

25

30

Then, each clone of the genomic library in E.coli prepared above as the donor is grown in a liquid medium. Simultaneously a strain of Gluconobacter as a recipient is cultured in a test tube containing a suitable medium to log to early stationary phase. The broth of Gluconobacter is then mixed with each broth of E.coli at the ratio of about 1:10 to about 10:1. Each

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mixture is spotted on a nitrocellulose filter on the surface of agar plate of suitable medium for growth of both *Gluconobacter* and *E.coli*, and the cells on the filter are incubated for 3 hours to 7 days.

5

(3) The transconjugants can be selected from the mixture of the donor *E.coli*, non-transconjugated *Gluconobacter* and transconjugated *Gluconobacter* by appropriate methods, for example, selection by antibiotic resistance and amino acid requirements.

10

(4) The genomic library in a strain of *Gluconobacter* can be prepared by collecting appropriate numbers of the above transconjugants.

15

Transconjugants can be also obtained by tri-parental conjugal mating using *E.coli* harboring a recombinant plasmid as a donor, *E.coli* harboring helper plasmid having *Tra* genes such as RK2 or pRK2013 as a helper and a strain of *Gluconobacter* as a recipient.

20

The *Gluconobacter* strain used as a recipient herein may be any strain belonging to the genus *Gluconobacter*. Particularly a 2-KGA producer such as *Gluconobacter oxydans* IFO 3292, *Gluconobacter oxydans* IFO 3293 (FERM-P No.8356), *Gluconobacter oxydans* IFO 3294 and their derivatives, a L-sorbose accumulator such as *Gluconobacter oxydans* OX-4, or a high 2-KGA producer such as *Gluconobacter oxydans* N44-1 or *Gluconobacter oxydans* U-13 (FERM-BP No.1269) and their derivatives can be used. These derivative strains can be obtained by usual mutagenesis as described before.

30

C) Screening

In general, a genomic library can be screened by using the following methods:

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- 14 -

- (1) Colony hybridization of a genomic library with an oligonucleotide probe synthesized according to an appropriate amino acid sequence of the enzyme.
- 5 (2) Immunological screening of a genomic library by using an antibody against the enzyme as an antigen.
- (3) Direct expression screening of a genomic library in a suitable host.

10

In the present invention, the direct expression screening is preferably used by examining the genomic library constructed in *Gluconobacter* according to the above explanations. The direct expression screening can be
15 performed by the procedure described hereafter.

Each strain of a genomic library in *Gluconobacter* is grown on an agar plate, which contains appropriate antibiotics useful as a marker, at 10° to 40°C, preferably
20 at 25° to 30°C, for 1 to 7 days. Thus obtained cells are subjected to a resting-system where cells are suspended in the reaction mixture containing 1 to 5% of L-sorbose or L-sorbose, 0.3% NaCl and 1% CaCO₃, and incubated at 10° to 40°C, preferably at 25° to 30°C, for 1 to 7 days. The
25 resulting reaction mixture is applied to thin layer chromatography to detect positive clones. The positive clones are selected by the accumulation of 2-KGA from L-sorbose or L-sorbose, or when an L-sorbose accumulator strain is used, selected by the consumption of L-sorbose
30 accumulated from L-sorbose.

The above cloned DNA encoding coenzyme independent L-sorbose dehydrogenase can be characterized by restriction mapping and/or nucleotide sequencing according to methods
35 well known in the art.

- 15 -

D) Stability of the recombinant plasmid

As it is well known in the field of recombinant DNA technology, most of the recombinant microorganisms need some selective pressure such as antibiotics to prevent a loss of the foreign plasmid. However, addition of antibiotics or other drugs to the fermentation broth should be avoided when the products of such a fermentation concern foods, drinks or pharmaceutical products. It should also be avoided for the simplification of the process. Therefore, it has been tried to improve the stability of the recombinant system by particular techniques, e.g. the introduction of a par region into the plasmid, use of a low-copy-number plasmid, or controlled expression of a gene by temperature shift. induction with IPTG (isopropylthiogalactoside), etc.

It is known that the stability of a plasmid is dependent on the combination of a host organism and a plasmid.

It has now been found that the recombinant plasmids which are derived from the plasmids pVK100 and pVK102 are stable in a strain of Gluconobacter in any cultivations, for example, in MB medium, FB medium and the production medium for 2-KGA fermentation as well as pVK100 and pVK102 per se.

Thus, the present invention provides also recombinant plasmids stable in a strain of Gluconobacter, which plasmids are useful for the production of the coenzyme independent L-sorbose dehydrogenase in Gluconobacter, and for the production of 2-KGA from D-sorbitol, L-sorbose or L-sorbose.

The stable recombinant expression plasmid of the present invention is characterized in that consisting of at least a DNA fragment derived from the plasmid pVK100 or pVK102, a structural gene to be expressed in a strain of Gluconobacter and, if desired, an expression control region functionally.

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conjugated to said structural gene.

E) Transformed microorganism

5 The transformant capable of expressing the objective enzyme can be obtained by introducing the above cloned plasmid containing the structural gene of the enzyme into an appropriate host organism. When the plasmid does not have a proper expression control region for the structural gene, an
10 expression plasmid may be reconstructed by introducing a promotor sequence and SD (Shine-Dalgarno) sequence upstream of the structural gene functionally.

 In the Example, a DNA fragment containing both an
15 expression control region and the structural gene of the enzyme which was cloned from a strain of Gluconobacter is exemplified. In this case, a plasmid to which the DNA fragment is inserted can express the structural gene of the objective enzyme in a strain of Gluconobacter or an
20 appropriate organism as a host organism.

Production of L-sorbose dehydrogenase using the transformed microorganism

25 A) Production and isolation

 The transformed microorganism obtained above may be utilized for the production of L-sorbose dehydrogenase by a fermentation and isolation procedure.

30

 The conditions of the fermentation of the transformant may be selected as usual depending on the host strain. When the host organism is a strain of Gluconobacter, the fermentation condition as described before may also be applied to
35 the transformant.

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Isolation of L-sorbose dehydrogenase produced by the transformant may be carried out by the method well known in the field of protein chemistry.

5 B) Comparison of the recombinant product with the natural product

The verification of the identity of L-sorbose dehydrogenase produced by the recombinant organism with the
10 natural one can be carried out by an immunological test, which is well known in the art, as well as the comparison of catalytic properties, such as optimum pH, optimum temperature, molecular weight, thermostability, etc. of the two enzymes.

15

Thus, the L-sorbose dehydrogenase produced by the recombinant techniques of the present invention is useful as a catalyst for the production of 2-KGA from L-sorbose. The production of 2-KGA from L-sorbose can be effected not
20 only by the said enzyme, but also by the cell free extract, resting cells or the growing cells of the recombinant organism.

The recombinant organism is also useful for the
25 production of 2-KGA from D-sorbitol or L-sorbose in a resting cell system or in a growing cell system.

Typical conditions for the conversion from L-sorbose to 2-KGA using the isolated recombinant enzyme or the cell
30 free extract are the same as that for the procedure for natural L-sorbose dehydrogenase described before.

The recombinant organism of the present invention has considerable advantages over its parent organism in the
35 2-KGA production from D-sorbitol or L-sorbose, when the host organism is a strain of Gluconobacter. The recombinant organism may be cultured in an aqueous medium supplemented

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with appropriate nutrients under aerobic conditions. In the cultivation, D-sorbitol or L-sorbose used as the starting material can be added into the medium at an appropriate time of fermentation, preferably at the starting point. The concentration range of the starting material, such as D-sorbitol is at a level of 10-400g/L and the culture is maintained at about 10°C to 40°C, preferably at 25°C to 30°C. The cultivation may be conducted at pH values of about 4.0-8.0, preferably about 5.5-7.5. The cultivation period varies depending upon the recombinant organisms and nutrient medium to be used, it is preferably about 20 to 200 hours.

The present invention is further illustrated by the following examples.

Example 1

Production of 2-KGA from L-sorbose by Gluconobacter oxydans strains

G. oxydans strains IF03292, IF03294, IF012257, IF012258, ATCC 9937 and IF03293 (FERM-P No.8356) grown on No.4 (5g/L of glycerol, 5g/L of yeast extract, 5g/L of $MgSO_4 \cdot 7H_2O$) agar plates were inoculated into 5ml of No.3 B.G medium containing 70g/L of glycerol, 15g/L of yeast extract, 2.5g/L of $MgSO_4 \cdot 7H_2O$ and 10g/L of $CaCO_3$ in a test tube. After the incubation for 3 days at 30°C on a test tube shaker, one ml of the resulting culture was used to inoculate 50ml of the fresh No.3 B.G medium in a 500ml-Erlenmeyer flask. The flasks were incubated for 3 days at 30°C on a rotary shaker operating at 180rpm. 20ml of each culture was centrifuged at 5000rpm for 15 minutes. The solids containing cells and remaining $CaCO_3$ were collected, washed twice with 10ml of sterile 0.3% NaCl and suspended in 6ml of the reaction solution containing 3g/L of NaCl, 30g/L of L-sorbose and 10g/L of $CaCO_3$. The reaction was conducted in a test tube

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for 23 hours at 30°C on a tube shaker. The yield of 2-KGA are summarized in Table 1. Evaporation loss during cultivation was not corrected.

5

Example 2Isolation and purification of L-sorbose dehydrogenase from Gluconobacter oxydans IF01225810 (1) Cultivation of G. oxydans IF012258

The agar slant culture of G. oxydans IF012258 was inoculated into 5ml of the medium composed of 70g/L of glycerol, 15g/L of yeast extract (Oriental Co., Ltd.) and 2.5g/L of $MgSO_4 \cdot 7H_2O$ in a test tube, and incubated at 30°C
15 for 2 days on a tube shaker (280r.p.m.). Two ml of this culture were transferred to 100ml of the same medium in a 500ml Erlenmeyer flask, and cultivated at 30°C for 20 hours on a rotary shaker (180r.p.m.). The culture thus prepared was used as an inoculum for a 30L jar fermentor containing
20 20L of the same medium. The jar fermentor was operated at 30°C, 250r.p.m. (for agitation) and 20L/min. (for aeration). After 40 hours of fermentation, the culture was harvested to collect the cells by centrifugation (8,000r.p.m.). From 20L of broth, 500g (wet weight) of the cells were obtained. The
25 cells were frozen at -20°C until use.

(2) Preparation of the membrane fraction

The frozen cells of G. oxydans IF012258 (500g, wet weight) were thawed and suspended in 2,500ml of 0.85% NaCl
30 solution. The cell suspension was then homogenized by a Dyno Mill homogenizer (Willy A. Bachofen Co., Basle) in the presence of glass beads (0.1mm in diameter) at 2,000r.p.m. for 4 minutes at 4°C.

35 The homogenate thus prepared was centrifuged at 1,800 g for 10 minutes to remove the cell debris and glass beads. The resulting supernatant was centrifuged at 80,000 x g for

- 20 -

60 minutes, and then the precipitate was collected as membrane fraction (200g, wet weight).

5 (3) Solubilization of L-sorbose dehydrogenase from the membrane fraction

The membrane fraction (200g, wet weight) was suspended in 900ml of 50mM potassium phosphate buffer (pH 7) containing 1% Triton X-100, stirred for 15 hours and centrifuged at 80,000 x g for 1 hour to obtain the
10 supernatant (850ml).

(4) DEAE(diethylamino ethyl)-Toyoparl(polyvinyl type) 650S (Toyo Soda) column chromatography

The supernatant (330ml) thus obtained was dialyzed
15 against 20L of 1mM potassium phosphate buffer containing 0.1% Triton X-100 for 15 hours and applied to a DEAE-Toyoparl 650S column (2.3 x 40cm), which had been equilibrated with the same buffer. The column was washed with the same buffer, and the enzyme was eluted by a linear
20 gradient of NaCl from 0.0M to 0.4M.

(5) DEAE-Sepharose CL-6B column chromatography

The active fractions from the previous step were combined and dialyzed against 1mM potassium phosphate buffer
25 containing 0.1% Triton X-100 for 15 hours, and then applied to a DEAE-Sepharose CL-6B column (2.5 x 10cm) equilibrated with the buffer. The column was washed with the same buffer, and the enzyme was eluted by a linear gradient of NaCl from 0.0M to 0.4M.

30

(6) CM-Sepharose CL-6B column chromatography

The active fractions from the previous step were combined and dialyzed against 1.0mM acetate buffer, pH5.5, containing 0.1% Triton X-100, for 15 hours, and then applied
35 to a CM-Sepharose CL-6B column (2.5 x 14.5cm) equilibrated with the same buffer. The column was washed with the same buffer, and the enzyme was eluted by a linear gradient of

- 21 -

NaCl from 0.0M to 0.4M.

(7) Hydroxyapatite HCA 100S column chromatography

The active fractions from the previous step were
5 combined and dialyzed against 1.0mM potassium phosphate
buffer (pH7.0) containing 0.1% Triton X-100, for 15 hours,
and then applied to a Hydroxyapatite HCA 100S column (1.7 x
18cm) equilibrated with the same buffer. The column was
washed with the same buffer, and the enzyme was eluted by
10 10mM potassium phosphate buffer (pH7.0) containing 0.1%
Triton X-100.

(8) TSK-GEL Toyopearl HW60S column (polyvinyl type)
chromatography

15 The active fractions from the previous step were
combined and concentrated by ultrafiltration using the
membrane filter (Diaflo PM-30, Amicon) to a small volume
(ca. 1.5ml). Then, the concentrate was applied to a
TSK-GEL Toyopearl HW60S column (1.5 x 80cm) equilibrated
20 with 50 mM potassium phosphate buffer containing 0.1% Triton
X-100. The column was developed by the same buffer.

A summary of the purification procedure of membrane-
bound L-sorbose dehydrogenase is shown in Table 2.

25

Example 3

Properties of L-sorbose dehydrogenase

30 (1) Electrophoretic analysis

The purified enzyme with a specific activity of 6.7
unit/mg protein was treated by sodium dodecyl sulfate (SDS)
and was analyzed for its purity by SDS-polyacrylamide
electrophoresis. It could be proved that the enzyme
35 consists of a single homogeneous subunit with a molecular
weight of $47,500 \pm 5,000$.

- 22 -

(2) Catalytic properties

The purified L-sorbose dehydrogenase showed its activity only when an electron acceptor such as 2,6-dichlorophenolindo-phenol or phenazine methosulphate was present.

The substrate specificity of the purified enzyme is shown in Table 3. Various types of aldehyde compounds were oxidized. Among them, methylglyoxal was oxidized most efficiently, and the reaction rate was in this case two times higher than that with L-sorbose.

The apparent Michaelis constant for L-sorbose was determined to be 16.7mM at pH7.0.

As shown in Table 4, the optimum temperature of L-sorbose dehydrogenase for methylglyoxal oxidation was found to be between 30°C and 40°C.

The effect of pH on L-sorbose oxidation is shown in Table 5. The enzyme showed its pH optimum at 7.0. The effect of metals on L-sorbose oxidation was tested. Among the metals tested, it was proved that Cu²⁺ inhibited the enzyme strongly (Table 6). Monoiodoacetic acid moderately inhibited the enzyme as shown in Table 7.

The pH stability of the purified enzyme was examined. After the enzyme was treated in the buffer of various pH's for 48 hours at 4°C, the residual enzyme activity was measured at pH7.0. As shown in Table 8, the enzyme was rather stable over the range of the pH's tested. About one third of the enzyme activity was lost at pH4.0.

The thermostability of L-sorbose dehydrogenase was examined. As shown in Table 9, about 70% of the enzyme activity was lost by the treatment of 40°C for 10 minutes.

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Example 4

Cloning of *G. oxydans* IF012258 L-sorbose dehydrogenase gene

5 (1) Construction of a cosmid genomic library in *E. coli* S17-1

(1)-a) Extraction of chromosomal DNA from *G. oxydans*
IF012258

G. oxydans IF012258 was cultivated in 200ml of mannitol
10 broth (MB) (25 g/L of mannitol, 3 g/L of bactopectone, 5g/L
of yeast extract) for 48 hours at 30°C. The cells were
collected by centrifugation, washed with 100ml of Tris
(10mM)-EDTA (1mM) buffer and resuspended in 50ml of Tris
(10mM)-EDTA (20mM) buffer.

15

The cell suspension thus prepared was treated with 2ml
of the lysozyme solution (10mg/ml) at 37°C for 30 minutes
followed by the treatment with pronase (4000 units) at 37°C
for 30 minutes and 10ml of 5% SDS at 37°C for 1 hour. At
20 this point, a clear lysate was obtained. The DNA was
extracted with 60ml of neutral phenol : chloroform
containing 4% octanol (1:1) by rotating slowly at 4°C for
30 minutes. The mixture was centrifuged at 15000rpm for
15 minutes and the supernatant obtained was extracted with
25 60ml of chloroform : octanol (96:4) by rotating slowly at
4°C for 10 minutes.

To the 50ml supernatant obtained by centrifugation at
15000rpm for 15 minutes was added 5.0ml of 3M sodium acetate
30 and then slowly 55ml of cold ethanol. The crude DNA was
obtained by winding out with a glass rod, which was then
treated with RNase T₁ and A (37°C, 30 minutes) and pronase
(37°C, 30 minutes) again. The phenol and chloroform
extractions were repeated to obtain pure chromosomal DNA.

35

(1)-b) Preparation of the vector plasmid

A cosmid vector, pVK102 (E.W. Nester et al, Plasmid 8,

- 24 -

45-54, (1982), was prepared from E. coli HB101 harboring pVK102 by the alkaline method (H.C. Birnboim and J. Doly, Nucleic Acids Research, 7, 1513-1523, 1979). The restriction map is illustrated in Fig. 1.

5

(1)-c) In vitro packaging

The total chromosomal DNA of G. oxydans IF012258 prepared in (1)-a) was partially digested with Sal I (Takara Shuzo Co., Ltd.). The resulting fragments of 15kb to 35kb
10 were isolated from agarose by gel electrophoresis. Plasmid pVK102 (ATCC 37158) prepared in (1)-b) was completely digested with Sal I and dephosphorylated with calf intestine alkaline phosphatase (Boehringer Mannheim GmbH). The DNA fragments of 15kb to 35kb and the linear pVK102 DNA were
15 ligated with T4 DNA ligase (Takara Shuzo Co., Ltd.).

The ligated fragments were used for the in vitro packaging using a packaging kit (Amersham International plc). The resulting phage particles were incubated with E.
20 coli ED8767 (Murray, N.E. et al, Mol. Gen. Genet. 150, 53, 1977). The cell suspension was plated onto LB agar plates containing 50 mg/L kanamycin.

(1)-d) Construction of the genomic library in E. coli
25 S17-1

One thousand colonies with the marker $Km^r Tc^s$ on the LB (Luria broth) agar plates containing 50mg/L kanamycin were scraped and used to prepare a mixture of recombinant plasmids. The plasmid DNAs were used to transform E. coli
30 S17-1 ($Sm^r Tra^+$) (constructed by Simon R. et al, Biotechnology 1, 784-791, 1982).

One thousand and four hundred transformants were picked into microtiter plates containing LB medium with 100µg/ml
35 of streptomycin and 50µg/ml of kanamycin, incubated overnight and stored with 15% glycerol at -80°C as the genomic library of G. oxydans IF012258 in E. coli S17-1.

The average size of the inserts was 25kb to 30kb.

(2) Conjugal transfer of the cosmid genomic library in
E. coli S17-1 into G. oxydans OX-4

5 The cosmid genomic library of G. oxydans IFO 12258 in
E. coli S17-1 was transferred to G. oxydans OX-4 a
L-sorbose accumulating mutant (obtained from G. oxydans
IFO 3293 by mutagenesis as described in European Patent
Publication No. 213 591) using bi-parental mating between
10 both strains.

Two hundred μ l of log phase culture of the recipient
G. oxydans OX-4 grown in mannitol broth were mixed with
100 μ l of log phase culture of every E. coli S17-1 carrying
15 pVK102 with insert DNA individually and spotted onto
nitrocellulose filter on the surface of FB (50g/L fructose,
5g/L yeast extract, 5g/L polypeptone) agar plates. The
plates were incubated overnight at 30°C. The mixed colonies
were streaked onto MB (mannitol broth) containing 10 μ g/ml
20 polymyxin B and 50 μ g/ml kanamycin (this medium being
termed MPK) agar plates and incubated for 4 days at 30°C.
The resulting conjugants were purified by restreaking on MPK
agar plates.

25 (3) Screening of the genomic library in G. oxydans OX-4

Screening was carried out using a mini-resting system.
About 1400 strains of G. oxydans OX-4/pVK102 with insert DNA
were suspended in the reaction mixture containing 50 μ l of
3g/L of NaCl, 10g/L of CaCO₃ and 30g/L of L-sorbose or L-
30 sorbose individually and incubated for 1 to 5 days at
30°C. Assays for 2-KGA production were performed by a
thin-layer chromatography of silica gel. One positive clone,
p7A6, was obtained.

35 (4) Subcloning of p7A6 and characterization of its subclones

The recombinant plasmid p7A6 was prepared by the
alkaline method of Birnboim and Doly (Nucleic Acids Research

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7. 1513-1523 (1979)) and fragmented with the following restriction enzymes:

EcoRI, EcoRV, HaeIII, HincII, NruI, Sal I, Sau 3A and
5 XhoII (Boehringer Mannheim GmbH), BamHI, BglII, Dra I, Hind
III and Sma I (Takara Shuzo Co., Ltd.). EcoRI, HincII, NruI
and Sal I digested the insert DNA of p7A6 (25kb) into 8 to
11 fragments. HaeIII, Sau 3A and XhoII generated numerous
number of fragments. Sal I was selected for the first step
10 of subcloning.

p7A6 was partially digested with Sal I, ligated with
dephosphorylated pVK102 digested with Sal I. The DNA
mixture was transferred to E. coli S17-1 by transformation
15 and then to G. oxydans OX-4 by bi-parental conjugation, as
described in Example 4.-(2). The smallest subclone,
p7A6Δ2, was isolated from the mini-resting screening of
200 conjugants and its size of the insert was 9.2kb
(Fig.2-(a)). A second subcloning was conducted by a deletion
20 of the fragment E₁-E₂ of p7A6Δ2. p7A6Δ3 (Fig.
2-(b)) thus obtained was further shortened by a deletion of
the fragment E_{1/2}-Sm₃. The resulting subclone, p7A6Δ4
(Fig. 2-(c)), contained a 3.1kb insert in pVK102 with a
small deletion of Sm₂-Sm₃ in the vector DNA. The
25 detailed restriction map of the 3.1kb S₁-S₂-E_{1/2} (SSE)
fragment is illustrated in Fig. 3.

(5) Production of recombinant L-sorbose dehydrogenase and
its characterization

30

p7A6 was introduced into G. oxydans N44-1 (such strain
being obtainable as outlined for G. oxydans OX-4) from E.
coli S17-1/p7A6 by a bi-parental conjugation, as described
in Example 4.-(2). The conjugant G. oxydans N44-1/p7A6 was
35 cultivated in 500ml of No.5 medium (100g/L L-sorbose, 15g/L
yeast extract, 2.5g/L MgSO₄·7H₂O, 0.5g/L glycerol and
20g/L CaCO₃) containing 50mg/L kanamycin at 30°C for 2

- 27 -

days. The cells thus obtained were disrupted by a French Press cell homogenizer in potassium phosphate buffer, pH7.0, treated with DNase in the presence of $MgCl_2$, and centrifuged (6000rpm, 15 min.). The supernatant was

5 centrifuged at 45000rpm (80,000 x g) for one hour to obtain a crude membrane fraction. L-sorbose dehydrogenase was solubilized from the membrane fraction with 2% Triton X-100 and partially purified through chromatofocusing (i.e. isoelectric chromatography: chromatographic separation

10 method of proteins based on the differences of the isoelectric points of the proteins) and hydroxylapatite column chromatography. SDS-PAGE analysis of the active protein which was detected by active staining and eluted from the PAGE gel exhibited one clear band at 47,500. Thus

15 the molecular weight of the subunit of L-sorbose dehydrogenase from N44-1/p7A6 was determined to be 47,500, identical with that of L-sorbose dehydrogenase isolated from *G. oxydans* IF012258 as described in Example 3-(1). About 300 μ g of the enzyme protein which had been eluted

20 from SDS-PAGE gel and precipitated with acetone was used to determine the partial amino acid sequence. A fifteen amino acid sequence of the N-terminus was determined as follows:

Met-Thr-Arg-Ser-Gln-Ile-Arg-Leu-Leu-Val-Ala-Thr-Thr-Ala-Val-

25

Furthermore, the proteolytic digest of the enzyme protein was chromatographed on HPLC.

Several peaks were separately collected and used to

30 determine the internal amino acid sequences of the protein.

Consequently, 6 partial amino acid sequences were determined as follows:

35 Arg-Asp-Thr-Asp-Gly-Asp-Gly-Ile-Ala-Asp-Gln-Arg,
Lys-Ala-Val-Asp-Leu-Pro-Ala-Gly-Tyr-Asn,
Arg-Ile-Asp-Arg-Phe-Asp-Ile-Ala-Thr-Gly-Lys,

- 28 -

Arg-Asn-Pro-Asn-Glu-Leu-Ala-Trp-Glu-Pro-Lys-Thr-Gly-
 Ala-Leu-Trp-Val-Ala-Val-Asn-Glu-Arg,
 Lys-Ser-Gly-Tyr-Arg-Val-Ile-Tyr-Val-Pro-Phe-Thr-
 Asp-Gly-His-Pro-Asp-Gly-Thr-Pro-Arg, and
 5 Arg-Val-Thr-Gly-Thr-Asp-Gln-Lys.

The allocation of these sequences on the L-sorbose dehydrogenase is indicated by the underlined portions in Figure 4.

10

The enzyme protein purified from the recombinant organism *G. oxydans* N44-1/p7A6 was also found to be identical with that of *G. oxydans* IF012258 immunologically.

15 (6) Sequencing of the SSE (SalI-SalII-EcoRI DNA-fragment containing L-sorbose dehydrogenase gene

Various fragments obtained from the SSE fragment (3.1kb) illustrated in Fig. 3 were further subcloned into the vector M13mp8 and M13mp9 (Messing, J. and Viera, J. Gene, 19, 20 269-276, 1982) and sequenced using the commercial sequencing kit (Amersham International plc) on both strands. Stepwise deletion (cutting off) with exonuclease III (Takara Shuzo Co., Ltd., Kilo sequencing kit) was employed.

25 7-Deaza-dGTP (2'-deoxyguanosine -5'-triphosphate) (Boehringer Mannheim GmbH) was used in place of dGTP. The N-terminus amino acid sequence of the enzyme protein was confirmed on the DNA sequence of the SSE fragment as follows:

30 5' ATG ACC CGT TCC CAG ATC AGG CTT CTC GTC GCG ACC ACC GCC
 GTC 3

Met Thr Arg Ser Gln Ile Arg Leu Leu Val Ala Thr Thr Ala
 Val

35

The open reading frame of 1347bp was found and its DNA sequence of the structure gene and amino acid sequence deduced from the DNA sequence are shown in Fig. 4.

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Example 5

Production of 2-KGA from L-sorbose by contacting purified
Gluconobacter oxydans IF012258 membrane-bound L-sorbose
5 dehydrogenase

The reaction mixture containing 100ml of purified
membrane-bound L-sorbose dehydrogenase (total activity,
130 units), 50ml of 0.5M potassium phosphate buffer (pH
10 7.0), 50ml of 10% L-sorbose solution, 10ml of 0.2M
phenazine methosulfate solution and 290ml of water was
incubated at 30°C with gentle shaking. As a result, 2-KGA
was formed with the rate of 650mg/hr.

15

Example 6

Production of 2-KGA from L-sorbose by recombinant
organisms under a resting-system

20 G. oxydans IF03292, G. oxydans IF03294, G. oxydans
IF03293, G. oxydans U-13 (FERM-BP No.1269) and their
conjugants carrying p7A6Δ4 prepared in the same manner as
described in Example 4-(5) were inoculated from MB agar
plate into 5ml of No.5 medium and incubated for 48 hours at
25 30°C. One ml of each culture was transferred to 50ml of the
same medium and these were cultivated at 30°C on a rotary
shaker (180rpm) for 48 hours. The remaining calcium
carbonate was removed by centrifugation at 500rpm for 5
minutes. The cells were collected, washed twice with 25ml
30 of sterile 3g/L NaCl solution, and suspended in 6ml of 3g/L
NaCl solution.

The reaction mixture containing 3g/L NaCl, 36g/L
L-sorbose, 10g/L CaCO_3 and 2ml cell suspension was
35 incubated in a test tube for 4 days at 30°C with shaking.
The amount of 2-KGA accumulated for 1 day or 4 days
incubation is shown in Table 10.

- 30 -

Example 7

Production of 2-KGA from L-sorbose by recombinant organisms under a resting-system

5

G. oxydans U-13 (FERM-BP No.1269) and G. oxydans U-13 containing p7A6Δ4 were used for the resting culture as described in Example 6 except that the substrate was 40g/L L-sorbose.

10

The yield of 2-KGA after 4 days incubation is shown in Table 11.

Example 8

15

Production of 2-KGA from L-sorbose by recombinant organism under a growing-system

Conjugants; G. oxydans N44-1 carrying pVK102 and
20 G. oxydans N44-1 carrying p7A6Δ4, were prepared in the same manner as described in Example 4-(2). Cells of each conjugant were inoculated from kanamycin-containing MB agar plate into 5ml of No.5 medium with or without kanamycin in a test tube and shaken for 48 hours at 30°C. One tenth ml of
25 each culture was transferred into 5ml of fresh No.5 medium with or without kanamycin in a test tube and shaken for 120 hours at 30°C.

G. oxydans N44-1, as a control, was cultivated in a
30 similar manner except that the cultivation was carried out without kanamycin.

The yield of 2-KGA is shown in Table 12.

35

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Example 9

Stability of recombinant plasmid during 2-KGA fermentation

5 Cells of transconjugant; *G. oxydans* N44-1 carrying
pVK102 or *G. oxydans* N44-1 carrying p7A6W4, were
inoculated from kanamycin-containing MB agar plate into 5ml
of No.5 medium without kanamycin in a test tube and shaken
for 48 hours at 30°C (the first cultivation). A 0.1ml
10 aliquot of each broth was transferred into 5ml of fresh No.5
medium without kanamycin in a test tube and shaken for 48
hours at 30°C (the second cultivation). The third
cultivation was carried out in the same manner as the second
cultivation except that the fermentation continued for 120
15 hours. Six samples were appropriately diluted after the
fermentation, spread on MB agar plates and incubated for 5
days at 30°C. The resulting colonies were picked up (50
colonies per one sample), streaked on MB agar plate with and
without kanamycin and incubated for 3 days at 30°C.

20

The stability of the plasmids was calculated as follows:

$$\text{Stability (\%)} = \frac{\text{Number of colonies with kanamycin-resistance}}{\text{Number of colonies picked up (50 colonies)}} \times 100$$

25

The result is shown in Table 13.

30

35

- 32 -

Table 12-KGA production from L-sorbose by *G. oxydans* strains

5

Strains		2-KGA (g/L)
10	<i>G. oxydans</i> IF03292	7.6
	IF03294	6.8
	IF012257	28.5
	IF012258	30.8
	ATCC9937	2.3
15	IF03293 (FERM-PNo.8356)	2.7

The initial concentration of L-sorbose was 30g/L.

20

Table 2Purification of L-sorbose dehydrogenase from
Gluconobacter oxydans IFO 12258

25

Fraction		Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
30	Solubilized fraction	2772	-	-	-
	DEAE-Toyopearl 650S	440	22.8	0.05	100
	DEAE-Sepharose CL-6B	247.5	15.4	0.06	67.5
	CM-Sepharose CL-6B	4.5	10.0	2.22	43.9
35	Hydroxyapatite HCA 100S	2.4	6.1	2.54	2.68
	TSK-GEL Toyopearl HW60S	0.3	2.0	6.67	8.8

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Table 3

Substrate specificity of membrane-bound L-sorbose dehydrogenase from *G. oxydans* IFO 12258

5		
	<u>Substrate</u>	<u>Relative activity (%)</u>
	L-Sorbose	100.0
	Methylglyoxal	200.0
10	Glyoxal	108.0
	Glutaraldehyde	102.7
	Glyoxylic acid	63.2
	Glyceraldehyde	55.3
	Glycolaldehyde	55.3
15	Propionaldehyde	29.0
	Acetaldehyde	21.1
	L-Sorbose	0
	D-Glucose	0

20

Table 4

Optimum temperature of membrane-bound L-sorbose dehydrogenase from *G. oxydans* IFO 12258

25		
	<u>Temperature (°C)</u>	<u>Relative activity (%)</u>
	10.	40.2
	20	61.2
	30	100.0
	40	100.0
30	50	85.1
	60	0

As the substrate, methylglyoxal was used.

35

- 34 -

Table 5

Optimum pH of membrane-bound L-sorbose dehydrogenase.
of G. oxydans IFO 12258

5

pH	Buffer (0.1 M)	Relative activity (%)
6.0	Potassium phosphate	46.1
6.5	"	60.4
7.0	"	100.0
7.5	"	76.2
8.0	"	85.8
8.0	Tris-HCl	82.6
8.5	"	63.5

10

L-Sorbose was used as a substrate.

15

Table 6

Effect of metal ions on membrane-bound L-sorbose
dehydrogenase of G. oxydans IFO 12258

20

Metal ion	Concentration (mM)	Relative activity (%)
None	0	100.0
Cu ²⁺	10	0
Co ²⁺	10	60.8
Mn ²⁺	10	69.5
Ni ²⁺	10	82.6
Fe ³⁺	10	100.0
Zn ²⁺	10	100.0
Mo ⁶⁺	10	117.0

25

30

L-Sorbose was used as the substrate.

35

- 35 -

Table 7

Effect of inhibitors on membrane-bound L-sorbose dehydrogenase of *G. oxydans* IFO 12258

5

Inhibitors	Concentration (mM)	Relative activity (%)
None	0	100
NaN ₃	25	89.4
ICH ₂ COOH	10	64.3

10

L-Sorbose was used as the substrate.

15

Table 8

pH stability of membrane-bound L-sorbose dehydrogenase of *G. oxydans* IFO 12258

20

pH	Buffer	Relative activity (%)
4.0	Acetate	64
4.5	"	89
5.0	"	89
5.5	"	90
6.0	Potassium phosphate	92
6.5	"	100
7.0	"	100
7.5	"	103
8.0	Tris-HCl	110

25

30

35

The purified enzyme was kept at the indicated pH for 48 hours at 4°C, and the residual enzyme activity was measured at pH 7.0. L-Sorbose was used as the substrate.

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Table 9

Thermostability of membrane-bound L-sorbose dehydrogenase of *G. oxydans* IFO 12258

5

	<hr/>	
	Temperature (°C)	Relative activity (%)
10	4	100
	20	88.0
	30	86.2
	40	29.4
15	50	3.4
	60	0
<hr/>		

The purified enzyme was treated at the indicated 20 temperature for 10 minutes, and the residual enzyme activity was measured at pH 7.0. L-Sorbose was used as the substrate.

25

30

35

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Table 10

2-KGA production from L-sorbose by recombinant organisms under a resting-system

5

			2-KGA* (g/L)	
			1 day	4 days
Strains			plasmid	
10	G. oxydans	IF03292	None	6.9
			P7A6Δ4	32.1
15		IF03294	None	6.2
			P7A6Δ4	33.9
		IF03293	None	2.0
			P7A6Δ4	34.2
20	U-13		None	8.9
			P7A6Δ4	33.7

The initial concentration of L-sorbose was 36g/L

25

* Evaporation loss during incubation was corrected.

30

35

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Table 11

2-KGA production from L-sorbose by recombinant organisms
under a resting-system

5

Strains	plasmid	2-KGA* (g/L)
G. oxydans U-13 (FERM-BP No.1269)	None	19.0
	p7A6Δ4	35.0

10

15

The initial concentration of L-sorbose was 40g/L
* Loss through evaporation during incubation was
corrected.

Table 12

20 2-KGA production from L-sorbose by recombinant organism
under a growing-system

25

30

Plasmid	Km*	2-KGA (g/L)
pVK102	+	55.7
"	-	53.8
p7A6Δ4	+	63.0
"	-	64.3
-	-	56.5

35

*: kanamycin

Host: G. oxydans N44-1

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Table 13

Stability* of vector and recombinant plasmid
during 2-KGA fermentation (%)

5

Plasmid	Km	<u>1st cultivation</u>	<u>2nd cultivation</u>	<u>3rd cultivation</u>
		48 hours	48 hours	120 hours
10 pVK102	-	96	94	82
p7A6Δ4	-	94	100	88

15

*Stability(%)=Number of colonies with kanamycin-resistance x 100
Total number of colonies tested

20

25

30

35

Claims

1. The coenzyme independent L-sorbose dehydrogenase which acts on L-sorbose to produce 2-keto-L-gulonate and originating from a microorganism of the genus *Gluconobacter*, in homogeneous form.
2. L-sorbose dehydrogenase according to claim 1 with the following physico-chemical properties:
 - a) optimum pH: about 7.0,
 - b) optimum temperature: about 30°C to about 40°C,
 - c) molecular structure: consisting of one type of unit having a molecular weight of about 47,500±5,000 as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis,
 - d) thermostability: stable below 30°C, and
 - e) inhibition: by Cu^{2+} -ions.
3. Coenzyme independent L-sorbose dehydrogenase according to claim 1 or 2, which is a membrane-bound enzyme.
4. Coenzyme independent L-sorbose dehydrogenase according to claim 2 or 3, which is a membrane-bound enzyme of *Gluconobacter oxydans* IFO 12257, *Gluconobacter oxydans* IFO 12258 or a mutant thereof.
5. A process for producing a novel coenzyme independent L-sorbose dehydrogenase which comprises cultivating a microorganism belonging to the genus *Gluconobacter* or a mutant thereof which is capable of producing the novel L-sorbose dehydrogenase, in cells, disrupting the cells, isolating and purifying it from cell free extract of the

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disrupted cells, preferably the membrane fraction of microorganism.

6. A process according to claim 5, wherein said
5 microorganism is *Gluconobacter oxydans* IFO 12257,
Gluconobacter oxydans IFO 12258 or a mutant thereof.

7. A process for producing 2-keto-L-gulonic acid, which
comprises converting L-sorbose to 2-keto-L-gulonic acid by
10 contacting L-sorbose with the novel coenzyme independent
L-sorbose dehydrogenase from a microorganism belonging to
the genus *Gluconobacter* containing a coenzyme independent
L-sorbose dehydrogenase.

8. A process according to claim 7, wherein said
15 microorganism is *Gluconobacter oxydans* IFO 12257,
Gluconobacter oxydans IFO 12258 or a mutant thereof.

9. A process for producing 2-keto-L-gulonic acid, which
20 comprises converting L-sorbose to 2-keto-L-gulonic acid by
contacting L-sorbose with a microorganism belonging to
genus *Gluconobacter* containing the novel coenzyme independent
L-sorbose dehydrogenase.

10. A process according to claim 9, wherein said
25 microorganism is *Gluconobacter oxydans* IFO 12257,
Gluconobacter oxydans IFO 12258 or a mutant thereof.

11. DNA encoding a polypeptide having an activity of a
30 novel coenzyme independent L-sorbose dehydrogenase capable
of converting L-sorbose to 2-keto-L-gulonic acid.

12. DNA according to claim 11, which encodes a gene of
novel coenzyme independent L-sorbose dehydrogenase having
35 the following partial amino acid sequences:

- 42 -

Met-Thr-Arg-Ser-Gln-Ile-Arg-Leu-Leu-Val-Ala-Thr-Thr-Ala-Val;
(N terminal)

Arg-Asp-Thr-Asp-Gly-Asp-Gly-Ile-Ala-Asp-Gln-Arg,

5

Lys-Ala-Val-Asp-Leu-Pro-Ala-Gly-Tyr-Asn,

Arg-Ile-Asp-Arg-Phe-Asp-Ile-Ala-Thr-Gly-Lys,

10 Arg-Asn-Pro-Asn-Glu-Leu-Ala-Trp-Glu-Pro-Lys-Thr-Gly-Ala-Leu-
Trp-Val-Ala-Val-Asn-Glu-Arg,

Lys-Ser-Gly-Tyr-Arg-Val-Ile-Tyr-Val-Pro-Phe-Thr-Asp-Gly-His-
Pro-Asp-Gly-Thr-Pro-Arg, and

15

Arg-Val-Thr-Gly-Thr-Asp-Gln-Lys.

13. A 3.1kb DNA fragment which contains a gene encoding a
novel coenzyme independent L-sorbosone dehydrogenase capable
20 of converting L-sorbosone to 2-keto-L-gulonic acid.

14. A DNA fragment according to claim 12, wherein said
coenzyme independent L-sorbosone dehydrogenase has the
following partial amino acid sequences:

25

Met-Thr-Arg-Ser-Gln-Ile-Arg-Leu-Leu-/al-Ala'-Thr-Thr-Ala-Val,
(N terminal)

Arg-Asp-Thr-Asp-Gly-Asp-Gly-Ile-Ala-Asp-Gln-Arg,

30

Lys-Ala-Val-Asp-Leu-Pro-Ala-Gly-Tyr-Asn,

Arg-Ile-Asp-Arg-Phe-Asp-Ile-Ala-Thr-Gly-Lys,

35 Arg-Asn-Pro-Asn-Glu-Leu-Ala-Trp-Glu-Pro-Lys-Thr-Gly-Ala-Leu-
Trp-Val-Ala-Val-Asn-Glu-Arg,

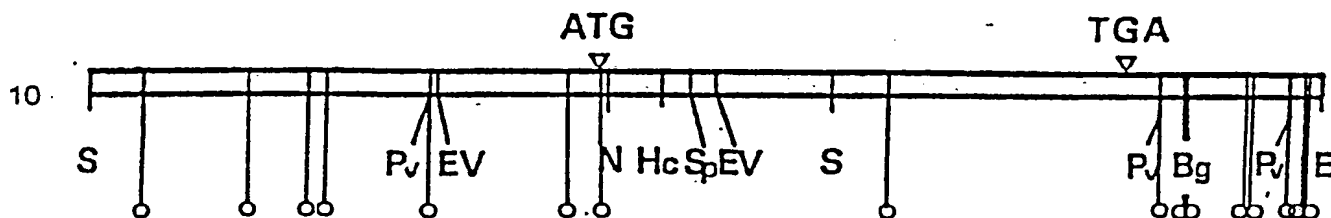
- 43 -

Lys-Ser-Gly-Tyr-Arg-Val-Ile-Tyr-Val-Pro-Phe-Thr-Asp-Gly-His-Pro-Asp-Gly-Thr-Pro-Arg, and

Arg-Val-Thr-Gly-Thr-Asp-Gln-Lys.

5

15. DNA fragment according to claim 13, which is characterized by the following restriction map:



15

— 200 bp

S : Sal I Pv: Pvu I EV: EcoRV N: Nru I Hc: Hinc II
 Sp: Sph I Bg: Bgl II E: EcoRI o: Sau3A

ATG: initiation codon TGA: stop codon

20

16. A recombinant DNA molecule which comprises a DNA sequence encoding a polypeptide having an activity of a novel coenzyme independent L-sorbose dehydrogenase capable of converting L-sorbose to 2-keto-L-gulonate.

25

17. A recombinant DNA molecule according to claim 16, wherein said coenzyme independent L-sorbose dehydrogenase has the following partial amino acid sequences:

30 Met-Thr-Arg-Ser-Gln-Ile-Arg-Leu-Leu-Val-Ala-Thr-Thr-Ala-Val,
 (N terminal)

Arg-Asp-Thr-Asp-Gly-Asp-Gly-Ile-Ala-Asp-Gln-Arg,

35 Lys-Ala-Val-Asp-Leu-Pro-Ala-Gly-Tyr-Asn,

Arg-Ile-Asp-Arg-Phe-Asp-Ile-Ala-Thr-Gly-Lys,

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Arg-Asn-Pro-Asn-Glu-Leu-Ala-Trp-Glu-Pro-Lys-Thr-Gly-Ala-Leu-
Trp-Val-Ala-Val-Asn-Glu-Arg,

Lys-Ser-Gly-Tyr-Arg-Val-Ile-Tyr-Val-Pro-Phe-Thr-Asp-Gly-His-
5 Pro-Asp-Gly-Thr-Pro-Arg, and

Arg-Val-Thr-Gly-Thr-Asp-Gln-Lys.

18. A recombinant DNA molecule according to claim 16,
10 which contains a sequence of a bacterial vector.

19. A recombinant DNA molecule according to claim 18,
wherein said bacterial vector is a cosmid vector.

15 20. A recombinant DNA molecule according to claim 19,
wherein said cosmid vector is the plasmid pVK100, pVK102 or a
derivative thereof.

21. A recombinant DNA molecule according to claim 16,
20 which is p7A6, p7A6Δ2, p7A6Δ3 or p7A6Δ4.

22. A recombinant DNA molecule stable in a microorganism
belonging to genus Gluconobacter which comprises a DNA
fragment derived from plasmid pVK100, pVK102 or a derivative
25 thereof.

23. A recombinant microorganism which has introduced a
recombinant DNA molecule having a DNA sequence encoding a
polypeptide having an activity of a novel coenzyme
30 independent L-sorbose dehydrogenase capable of converting
L-sorbose to 2-keto-L-gulononic acid.

24. A recombinant microorganism according to claim 23,
wherein said coenzyme independent L-sorbose dehydrogenase
35 has the following partial amino acid sequences:

- 45 -

Met-Thr-Arg-Ser-Gln-Ile-Arg-Leu-Leu-Val-Ala-Thr-Thr-Ala-Val,
(N terminal)

Arg-Asp-Thr-Asp-Gly-Asp-Gly-Ile-Ala-Asp-Gln-Arg,

5

Lys-Ala-Val-Asp-Leu-Pro-Ala-Gly-Tyr-Asn,

Arg-Ile-Asp-Arg-Phe-Asp-Ile-Ala-Thr-Gly-Lys,

10 Arg-Asn-Pro-Asn-Glu-Leu-Ala-Trp-Glu-Pro-Lys-Thr-Gly-Ala-Leu-
Trp-Val-Ala-Val-Asn-Glu-Arg,

Lys-Ser-Gly-Tyr-Arg-Val-Ile-Tyr-Val-Pro-Phe-Thr-Asp-Gly-His-
Pro-Asp-Gly-Thr-Pro-Arg, and

15

Arg-Val-Thr-Gly-Thr-Asp-Gln-Lys.

25. A recombinant microorganism according to claim 23 or
24, which belongs to the genus *Gluconobacter*.

20

26. A recombinant microorganism according to claim 25,
which is *Gluconobacter oxydans* OX-4/p7A6, *Gluconobacter*
oxydans N44-1/p7A6, *Gluconobacter oxydans* N44-1/p7A6Δ4,
Gluconobacter oxydans U-13/p7A6Δ4, *Gluconobacter oxydans*
25 IFO 3292/p7A6Δ4, *Gluconobacter oxydans* IFO 3293/p7A6Δ4 or
Gluconobacter oxydans IFO 3294/p7A6Δ4.

27. A recombinant microorganism according to any one of
claims 23 to 26, which is a transconjugant of a strain
30 belonging to the genus *Gluconobacter*.

28. A method for producing a transconjugant using a
microorganism belonging to the genus *Gluconobacter* as a
recipient, which comprises contacting said recipient with a
35 donor having a plasmid containing Mob site to transfer said
plasmid from said donor to said recipient with the help of
Tra gene function.

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29. A method according to claim 28, wherein said plasmid is pVK100, pVK102 or a derivative thereof.

30. A process for producing a polypeptide having an activity of a novel coenzyme independent L-sorbose dehydrogenase which comprises cultivating a microorganism having introduced a recombinant DNA molecule having a DNA sequence encoding a polypeptide having an activity of the L-sorbose dehydrogenase capable of converting L-sorbose to 2-keto-L-gulononic acid and disrupting the cells, isolating and purifying it from cell free extract of the disrupted cells, preferably from the membrane fraction of the microorganism.

31. A process according to claim 30, wherein said coenzyme independent L-sorbose dehydrogenase has the following partial amino acid sequences:

Met-Thr-Arg-Ser-Gln-Ile-Arg-Leu-Leu-Val-Ala-Thr-Thr-Ala-Val,
(N terminal)

Arg-Asp-Thr-Asp-Gly-Asp-Gly-Ile-Ala-Asp-Gln-Arg,

Lys-Ala-Val-Asp-Leu-Pro-Ala-Gly-Tyr-Asn,

Arg-Ile-Asp-Arg-Phe-Asp-Ile-Ala-Thr-Gly-Lys,

Arg-Asn-Pro-Asn-Glu-Leu-Ala-Trp-Glu-Pro-Lys-Thr-Gly-Ala-Leu-Trp-Val-Ala-Val-Asn-Glu-Arg,

Lys-Ser-Gly-Tyr-Arg-Val-Ile-Tyr-Val-Pro-Phe-Thr-Asp-Gly-His-Pro-Asp-Gly-Thr-Pro-Arg, and

Arg-Val-Thr-Gly-Thr-Asp-Gln-Lys.

32. A process according to claim 30, wherein said microorganism belongs to genus Gluconobacter.

33. A process according to any one of claims 30 to 32, wherein said microorganism is *Gluconobacter oxydans* OX-4/p7A6, *Gluconobacter oxydans* N44-1/p7A6, *Gluconobacter oxydans* N44-1/p7A6 Δ 4, *Gluconobacter oxydans* U-13/p7A6 Δ 4, 5 *Gluconobacter oxydans* IFO 3292/p7A6 Δ 4, *Gluconobacter oxydans* IFO 3293/p7A6 Δ 4 or *Gluconobacter oxydans* IFO 3294/p7A6 Δ 4.

34. A process for producing 2-keto-L-gulonic acid which 10 comprises converting L-sorbose to 2-keto-L-gulonic acid by contacting L-sorbose with a polypeptide having an activity of a novel coenzyme independent L-sorbose dehydrogenase from a microorganism introduced with a recombinant DNA molecule having a DNA sequence encoding said polypeptide.

15

35. A process according to claim 34, wherein said coenzyme independent L-sorbose dehydrogenase has the following partial amino acid sequences:

20 Met-Thr-Arg-Ser-Gln-Ile-Arg-Leu-Leu-Val-Ala-Thr-Thr-Ala-Val,
(N terminal)

Arg-Asp-Thr-Asp-Gly-Asp-Gly-Ile-Ala-Asp-Gln-Arg,

25 Lys-Ala-Val-Asp-Leu-Pro-Ala-Gly-Tyr-Asn,

Arg-Ile-Asp-Arg-Phe-Asp-Ile-Ala-Thr-Gly-Lys,

Arg-Asn-Pro-Asn-Glu-Leu-Ala-Trp-Glu-Pro-Lys-Thr-Gly-Ala-Leu- 30 Trp-Val-Ala-Val-Asn-Glu-Arg,

Lys-Ser-Gly-Tyr-Arg-Val-Ile-Tyr-Val-Pro-Phe-Thr-Asp-Gly-His-
Pro-Asp-Gly-Thr-Pro-Arg, and

35 Arg-Val-Thr-Gly-Thr-Asp-Gln-Lys.

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36. A process according to claim 34, wherein said microorganism belongs to genus *Gluconobacter*.

37. A process according to any one of claims 34 to 36,
5 wherein said microorganism is *Gluconobacter oxydans*
OX-4/p7A6, *Gluconobacter oxydans* N44-1/p7A6, *Gluconobacter*
oxydans N44-1/p7A6Δ4, *Gluconobacter oxydans* U-13/p7A6Δ4,
Gluconobacter oxydans IFO 3292/p7A6Δ4, *Gluconobacter*
oxydans IFO 3293/p7A6Δ4 or *Gluconobacter oxydans* IFO
10 3294/p7A6Δ4.

38. A process for producing 2-keto-L-gulonic acid which
comprises converting L-sorbose to 2-keto-L-gulonic acid
with the aid of a microorganism having introduced a
15 recombinant DNA molecule having a DNA sequence encoding a
polypeptide having an activity of a novel coenzyme
independent L-sorbose dehydrogenase capable of converting
L-sorbose to 2-keto-L-gulonic acid.

20 39. A process according to claim 38, wherein said
coenzyme independent L-sorbose dehydrogenase has the
following partial amino acid sequences:

Met-Thr-Arg-Ser-Gln-Ile-Arg-Leu-Leu-Val-Ala-Thr-Thr-Ala-Val,
25 (N terminal)

Arg-Asp-Thr-Asp-Gly-Asp-Gly-Ile-Ala-Asp-Gln-Arg,

Lys-Ala-Val-Asp-Leu-Pro-Ala-Gly-Tyr-Asn,
30

Arg-Ile-Asp-Arg-Phe-Asp-Ile-Ala-Thr-Gly-Lys,

Arg-Asn-Pro-Asn-Glu-Leu-Ala-Trp-Glu-Pro-Lys-Thr-Gly-Ala-Leu-
Trp-Val-Ala-Val-Asn-Glu-Arg,
35

Lys-Ser-Gly-Tyr-Arg-Val-Ile-Tyr-Val-Pro-Phe-Thr-Asp-Gly-His-
Pro-Asp-Gly-Thr-Pro-Arg, and

- 49 -

Arg-Val-Thr-Gly-Thr-Asp-Gln-Lys.

40. A process according to claim 38, wherein said microorganism belongs to the genus *Gluconobacter*.

5

41. A process according to any one of claims 38 to 40, wherein said microorganism is *Gluconobacter oxydans* OX-4/p7A6, *Gluconobacter oxydans* N44-1/p7A6, *Gluconobacter oxydans* N44-1/p7A6Δ4, *Gluconobacter oxydans* U-13/p7A6Δ4,
10 *Gluconobacter oxydans* IFO 3292/p7A6Δ4, *Gluconobacter oxydans* IFO 3293/p7A6Δ4 or *Gluconobacter oxydans* IFO 3294/p7A6Δ4.

42. A process for producing 2-keto-L-gulonic acid which
15 comprises converting L-sorbose to 2-keto-L-gulonic acid with the aid of a microorganism having introduced a recombinant DNA molecule having a DNA sequence encoding a polypeptide having an activity of a novel coenzyme independent
L-sorbose dehydrogenase capable of converting L-sorbose
20 to 2-keto-L-gulonic acid.

43. A process according to claim 42, wherein said coenzyme independent L-sorbose dehydrogenase has the following partial amino acid sequences:

25

Met-Thr-Arg-Ser-Gln-Ile-Arg-Leu-Leu-Val-Ala-Thr-Thr-Ala-Val,
(N terminal)

30

Arg-Asp-Thr-Asp-Gly-Asp-Gly-Ile-Ala-Asp-Gln-Arg,

Lys-Ala-Val-Asp-Leu-Pro-Ala-Gly-Tyr-Asn,

Arg-Ile-Asp-Arg-Phe-Asp-Ile-Ala-Thr-Gly-Lys,

35 Arg-Asn-Pro-Asn-Glu-Leu-Ala-Trp-Glu-Pro-Lys-Thr-Gly-Ala-Leu-
Trp-Val-Ala-Val-Asn-Glu-Arg,

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Lys-Ser-Gly-Tyr-Arg-Val-Ile-Tyr-Val-Pro-Phe-Thr-Asp-Gly-His-
Pro-Asp-Gly-Thr-Pro-Arg, and

Arg-Val-Thr-Gly-Thr-Asp-Gln-Lys.

5

44. A process according to claim 42, wherein said
microorganism belongs to genus *Gluconobacter*.

45. A process according to any one of claims 42 to 44,
10 wherein said microorganism is *Gluconobacter oxydans*
OX-4/p7A6, *Gluconobacter oxydans* N44-1/p7A6, *Gluconobacter*
oxydans N44-1/p7A6Δ4, *Gluconobacter oxydans* U-13/p7A6Δ4,
Gluconobacter oxydans IFO 3292/p7A6Δ4, *Gluconobacter*
oxydans IFO 3293/p7A6Δ4 or *Gluconobacter oxydans* IFO
15 3294/p7A6Δ4.

46. A process for producing 2-keto-L-gulonic acid which
comprises converting D-sorbitol to 2-keto-L-gulonic acid with
the aid of a microorganism having introduced a recombinant
20 DNA molecule having a DNA sequence encoding a polypeptide
having an activity of a novel coenzyme independent
L-sorbose dehydrogenase capable of converting L-sorbose
to 2-keto-L-gulonic acid.

25 47. A process according to claim 46, wherein said
coenzyme independent L-sorbose dehydrogenase has the
following partial amino acid sequences:

Met-Thr-Arg-Ser-Gln-Ile-Arg-Leu-Leu-Val-Ala-Thr-Thr-Ala-Val,
30 (N terminal)

Arg-Asp-Thr-Asp-Gly-Asp-Gly-Ile-Ala-Asp-Gln-Arg,

Lys-Ala-Val-Asp-Leu-Pro-Ala-Gly-Tyr-Asn,
35

Arg-Ile-Asp-Arg-Phe-Asp-Ile-Ala-Thr-Gly-Lys,

- 51 -

Arg-Asn-Pro-Asn-Glu-Leu-Ala-Trp-Glu-Pro-Lys-Thr-Gly-Ala-Leu-
Trp-Val-Ala-Val-Asn-Glu-Arg,

Lys-Ser-Gly-Tyr-Arg-Val-Ile-Tyr-Val-Pro-Phe-Thr-Asp-Gly-His-
5 Pro-Asp-Gly-Thr-Pro-Arg, and

Arg-Val-Thr-Gly-Thr-Asp-Gln-Lys.

48. A process according to claim 46, wherein said
10 microorganism belongs to genus *Gluconobacter*.

49. A process according to any one of claims 46 to 48,
wherein said microorganism is *Gluconobacter oxydans*
OX-4/p7A6, *Gluconobacter oxydans* N44-1/p7A6, *Gluconobacter*
15 *oxydans* N44-1/p7A6 Δ 4, *Gluconobacter oxydans* U-13/p7A6 Δ 4,
Gluconobacter oxydans IFO 3292/p7A6 Δ 4, *Gluconobacter*
oxydans IFO 3293/p7A6 Δ 4 or *Gluconobacter oxydans* IFO
3294/p7A6 Δ 4.

20 50. The DNA, the DNA fragments, the recombinant DNA
molecules, the recombinant microorganisms and the processes
according to claims 11, 14, 17, 31, 35, 38, 43 and 47,
wherein a functional equivalent of the partial amino acid
sequences involved is used.

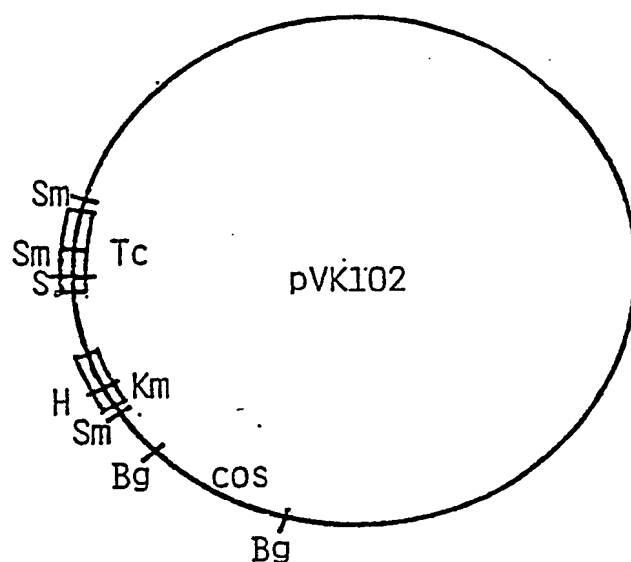
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35

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Fig.1 Restriction map of pVK102



S: Sal I
E: EcoRI
B: BamHI
Bg: BglII
H: HindIII
Sm: SmaI
Tc: Tetracycline resistance gene
Km: Kanamycin resistance gene
cos: cos site of λ phage

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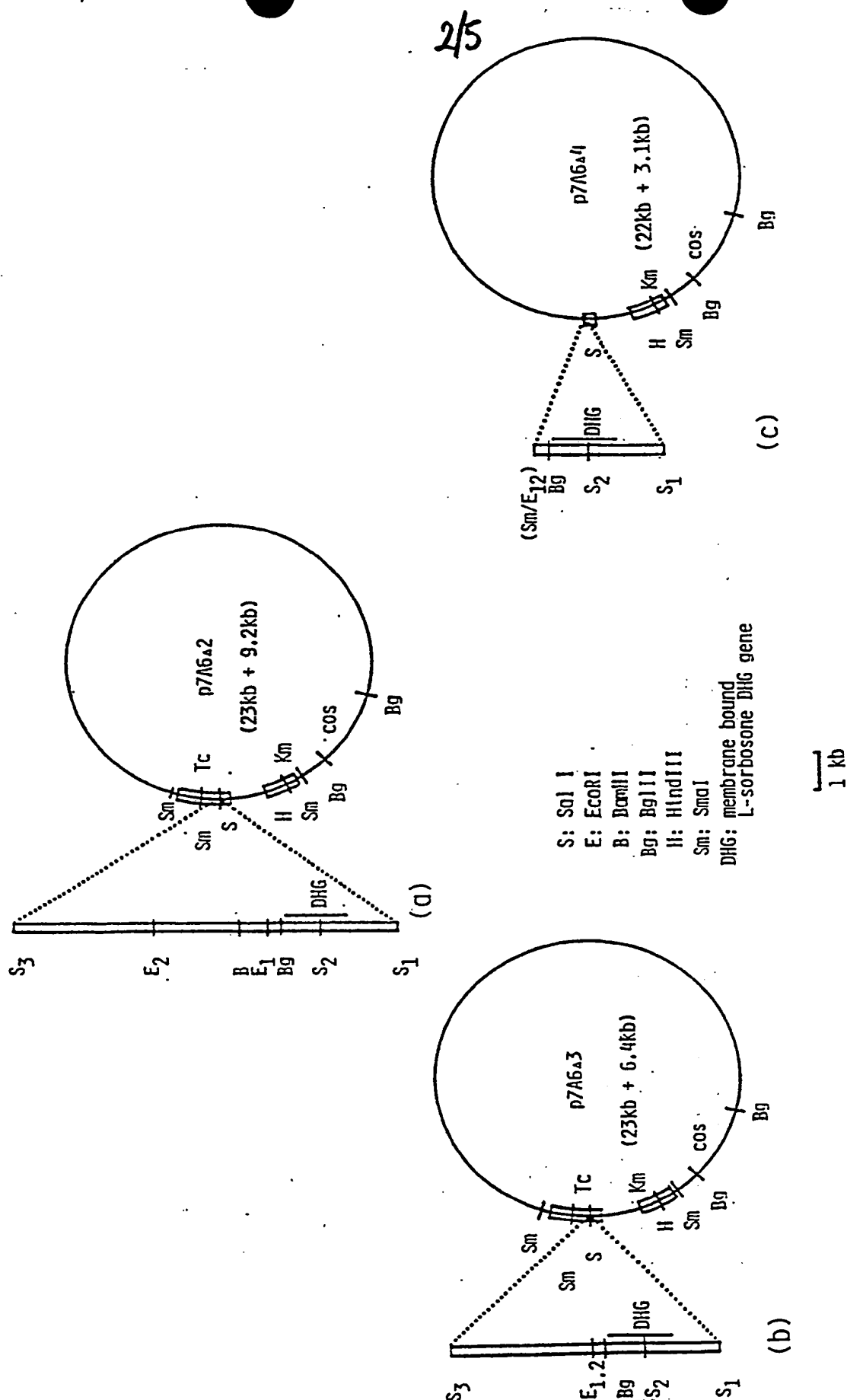


Fig. 2. Restriction map of the subclones

(a) p7A6 Δ 2 (b) p7A6 Δ 3 (c) p7A6 Δ 4

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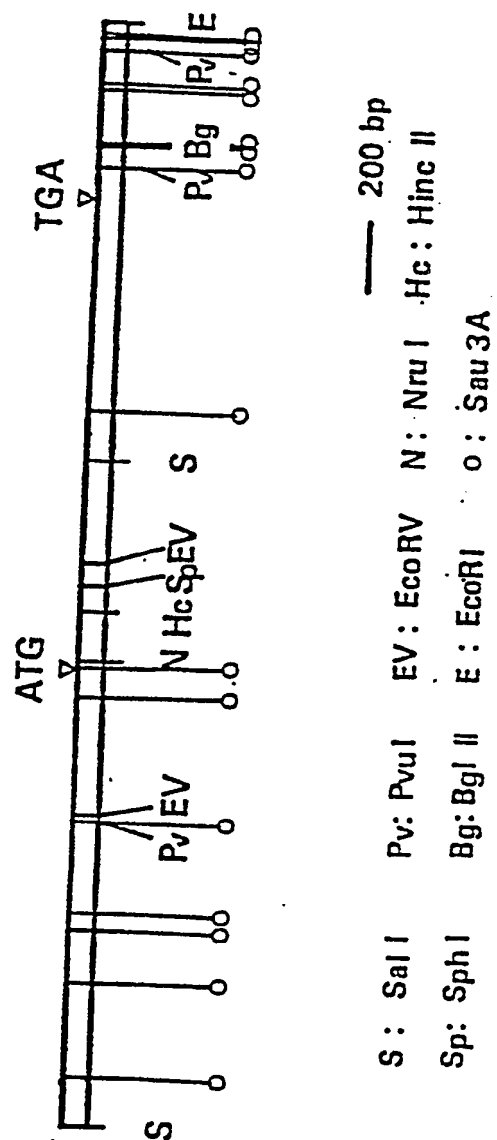


Fig. 3. Restriction map of SSE fragment (3.1 kb)

ATG: initiation codon TGA: stop codon

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1	ATG	ACC	CGT	TCC	CAG	ATC	AGG	CTT	CTC	GTC	GCG	ACC	ACC	GCC	GTC	ACC	48
1	Met	Thr	Arg	Ser	Gln	Ile	Arg	Leu	Leu	Val	Ala	Thr	Thr	Ala	Val	Thr	16
49	GCG	CTG	CTG	GTG	GCA	GCC	GGC	TAC	CGC	GCG	GTC	GTC	TCG	CCC	GAG	GAA	96
17	Ala	Leu	Leu	Val	Ala	Ala	Gly	Tyr	Arg	Ala	Val	Val	Ser	Pro	Glu	Glu	32
97	GCC	CGG	CAG	ACG	GTC	GCG	GCC	GGA	ACC	GGC	CCC	CAC	CCC	GTC	CTG	CCG	144
33	Ala	Arg	Gln	Thr	Val	Ala	Ala	Gly	Thr	Gly	Pro	His	Pro	Val	Leu	Pro	48
145	CCG	CCC	AAC	CCC	ACC	TTC	ATG	CCC	ACG	GTC	AAC	ATC	GCC	ACG	CCC	GTC	192
49	Pro	Pro	Asn	Pro	Thr	Phe	Met	Pro	Thr	Val	Asn	Ile	Ala	Thr	Pro	Val	64
193	GGC	TGG	CAG	GGC	ACG	CAG	GCC	CCG	ACC	CCG	GCG	GCG	GGG	CTG	GCG	GTG	240
65	Gly	Trp	Gln	Gly	Thr	Gln	Ala	Pro	Thr	Pro	Ala	Ala	Gly	Leu	Ala	Val	80
241	CAT	GCC	TTC	GCC	ACC	GGC	CTG	GAC	CAC	CCC	CGC	TGG	CTG	TAC	AAG	CTG	288
81	His	Ala	Phe	Ala	Thr	Gly	Leu	Asp	His	Pro	Arg	Trp	Leu	Tyr	Lys	Leu	96
289	CCC	AAC	GGC	GAT	ATC	CTG	GTG	GCG	GAA	TCC	GAG	TCC	CCC	GGC	ACC	GAC	336
97	Pro	Asn	Gly	Asp	Ile	Leu	Val	Ala	Glu	Ser	Glu	Ser	Pro	Gly	Thr	Asp	112
337	ATC	AAG	ACG	GTG	AAG	AAC	CGC	ATC	GCC	GGC	CTG	GTC	ATG	GGC	CAG	GTC	384
113	Ile	Lys	Thr	Val	Lys	Asn	Arg	Ile	Ala	Gly	Leu	Val	Met	Gly	Gln	Val	128
385	GGC	GCG	GGC	GGA	AAA	AGC	CCC	GAC	CGC	ATC	ATC	CTG	CTG	CGC	GAT	ACC	432
129	Gly	Ala	Gly	Gly	Lys	Ser	Pro	Asp	Arg	Ile	Ile	Leu	Leu	Arg	Asp	Thr	144
433	GAC	GGC	GAC	GGC	ATC	GCC	GAC	CAG	CGC	AGC	GTG	TTC	CTC	GAC	CAC	CTC	480
145	Asp	Gly	Asp	Gly	Ile	Ala	Asp	Gln	Arg	Ser	Val	Phe	Leu	Asp	His	Leu	160
481	TAC	TCG	CCC	TTC	GGC	ATG	GCG	CTG	GTC	GGC	GAC	ACG	CTC	TAC	GTG	GCC	528
161	Tyr	Ser	Pro	Phe	Gly	Met	Ala	Leu	Val	Gly	Asp	Thr	Leu	Tyr	Val	Ala	176
529	AAC	GCC	AAC	GCG	CTG	GTC	CGC	TTC	CCC	TAT	CAC	GAG	GGC	GAA	ACC	CAC	576
177	Asn	Ala	Asn	Ala	Leu	Val	Arg	Phe	Pro	Tyr	His	Glu	Gly	Glu	Thr	His	192
577	ATC	GAC	GCA	CCG	GGC	GAG	AAA	GCC	GTC	GAC	CTC	CCG	GCC	GGC	TAC	AAC	624
193	Ile	Asp	Ala	Pro	Gly	Glu	Lys	Ala	Val	Asp	Leu	Pro	Ala	Gly	Tyr	Asn	208
625	CAC	CAC	TGG	ACC	AAG	AAC	ATC	CTG	GCC	AGC	CCG	GAC	GGC	AGC	ACC	CTC	672
209	His	His	Trp	Thr	Lys	Asn	Ile	Leu	Ala	Ser	Pro	Asp	Gly	Ser	Thr	Leu	224
673	TAC	GTG	ACC	GTC	GGC	TCC	AAC	AGC	AAC	GTC	GCC	GAC	AAC	GGC	ATG	GAG	720
225	Tyr	Val	Thr	Val	Gly	Ser	Asn	Ser	Asn	Val	Ala	Asp	Asn	Gly	Met	Glu	240
721	GTC	GAG	GAA	GGC	CGC	GCC	CGG	ATC	GAC	CGG	TTC	GAC	ATC	GCC	ACC	GGC	768
241	Val	Glu	Glu	Gly	Arg	Ala	Arg	Ile	Asp	Arg	Phe	Asp	Ile	Ala	Thr	Gly	256
769	AAG	CTC	ACC	CCC	TAC	GCC	ACC	GGC	CTG	CGC	AAC	CCC	AAC	GAG	CTG	GCG	816
257	Lys	Leu	Thr	Pro	Tyr	Ala	Thr	Gly	Leu	Arg	Asn	Pro	Asn	Glu	Leu	Ala	272

Fig. 4

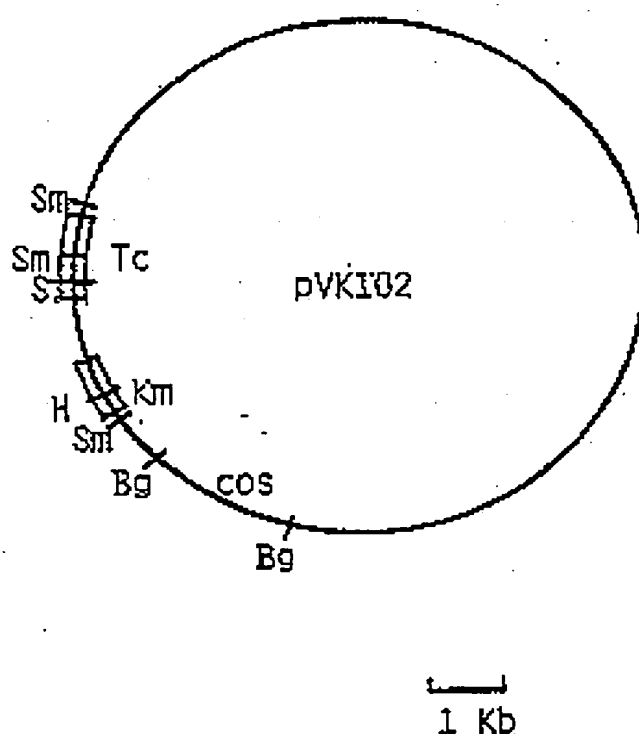
5/5

817	TGG	GAG	CCC	AAG	ACC	GGC	GCC	CTG	TGG	GTC	GCA	GTG	AAC	GAA	CGC	GAC	864
273	<u>Trp</u>	<u>Glu</u>	<u>Pro</u>	<u>Lys</u>	<u>Thr</u>	<u>Gly</u>	<u>Ala</u>	<u>Leu</u>	<u>Trp</u>	<u>Val</u>	<u>Ala</u>	<u>Val</u>	<u>Asn</u>	<u>Glu</u>	<u>Arg</u>	<u>Asp</u>	288
865	GAA	ATC	GGC	AGC	GAC	CTG	GTG	CCC	GAC	TAC	ATC	ACG	GCG	GTG	AAG	GAG	912
289	Glu	Ile	Gly	Ser	Asp	Leu	Val	Pro	Asp	Tyr	Ile	Thr	Ala	Val	Lys	Glu	304
913	GGC	GCG	TTG	TAC	GGC	TGG	CCC	TAC	AGC	TAT	TAC	GGC	CAG	CAT	GTC	GAT	960
305	Gly	Ala	Phe	Tyr	Gly	Trp	Pro	Tyr	Ser	Tyr	Tyr	Gly	Gln	His	Val	Asp	320
961	GTC	CGC	GTC	AAG	CCG	CAG	CGG	CCC	GAC	CTG	GTG	GCC	AGC	GCC	ATC	GCC	1008
321	Val	Arg	Val	Lys	Pro	Gln	Arg	Pro	Asp	Leu	Val	Ala	Ser	Ala	Ile	Ala	336
1009	CCC	GAC	TAC	GCG	CTC	GGC	CCG	CAC	ACC	GCC	TGG	TTT	GGC	ATC	GCC	TTC	1056
337	Pro	Asp	Tyr	Ala	Leu	Gly	Pro	His	Thr	Ala	Trp	Phe	Gly	Ile	Ala	Phe	352
1057	TCG	CAG	GAC	AGC	AGC	CTG	CCC	GCG	GCC	TGG	CGC	AAT	GGC	CTG	TTC	GTC	1104
353	Ser	Gln	Asp	Ser	Ser	Leu	Pro	Ala	Ala	Trp	Arg	Asn	Gly	Leu	Phe	Val	368
1105	GCC	CAG	CAC	GGC	TCA	TGG	AAC	CGC	AAG	CCC	AAG	AGC	GGC	TAC	CGC	GTC	1152
369	Ala	Gln	His	Gly	Ser	Trp	Asn	Arg	Lys	Pro	<u>Lys</u>	<u>Ser</u>	<u>Gly</u>	<u>Tyr</u>	<u>Arg</u>	<u>Val</u>	384
1153	ATC	TAC	GTC	CCC	TTC	ACC	GAC	GGC	CAC	CCC	GAC	GGC	ACC	CCC	CGC	GAG	1200
385	<u>Ile</u>	<u>Tyr</u>	<u>Val</u>	<u>Pro</u>	<u>Phe</u>	<u>Thr</u>	<u>Asp</u>	<u>Gly</u>	<u>His</u>	<u>Pro</u>	<u>Asp</u>	<u>Gly</u>	<u>Thr</u>	<u>Pro</u>	<u>Arg</u>	<u>Asp</u>	400
1201	GTG	CTG	ACC	GGC	TTC	CTC	ACA	CAG	GAC	GAA	GAC	CAC	GCC	CAC	GGC	CGC	1248
401	Val	Leu	Thr	Gly	Phe	Leu	Thr	Gln	Asp	Glu	Asp	His	Ala	His	Gly	Arg	416
1249	CCG	GTC	GGC	CTG	GCG	CTG	GAC	AAA	TCC	GGC	GCC	CTC	CTG	GTC	GCC	GAC	1296
417	Pro	Val	Gly	Leu	Ala	Leu	Asp	Lys	Ser	Gly	Ala	Leu	Leu	Val	Ala	Asp	432
1297	GAT	GTC	GGC	AAC	ACC	GTG	TGG	CGC	GTC	ACC	GGC	ACG	GAC	CAG	AAG	ACC	1344
433	Asp	Val	Gly	Asn	Thr	Val	Trp	<u>Arg</u>	<u>Val</u>	<u>Thr</u>	<u>Gly</u>	<u>Thr</u>	<u>Asp</u>	<u>Gln</u>	<u>Lys</u>	<u>Thr</u>	448
1345	GAC																
449	Asp																

Fig. 4 DNA sequence of L-sorbose dehydrogenase of
G. oxdans IF012258

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Fig.1 Restriction map of pVK102



- S: Sal I
E: EcoRI
B: BamHI
Bg: Bgl II
H: Hind III
Sm: Sma I
Tc: Tetracycline resistance gene
Km: Kanamycin resistance gene
cos: cos site of λ phage

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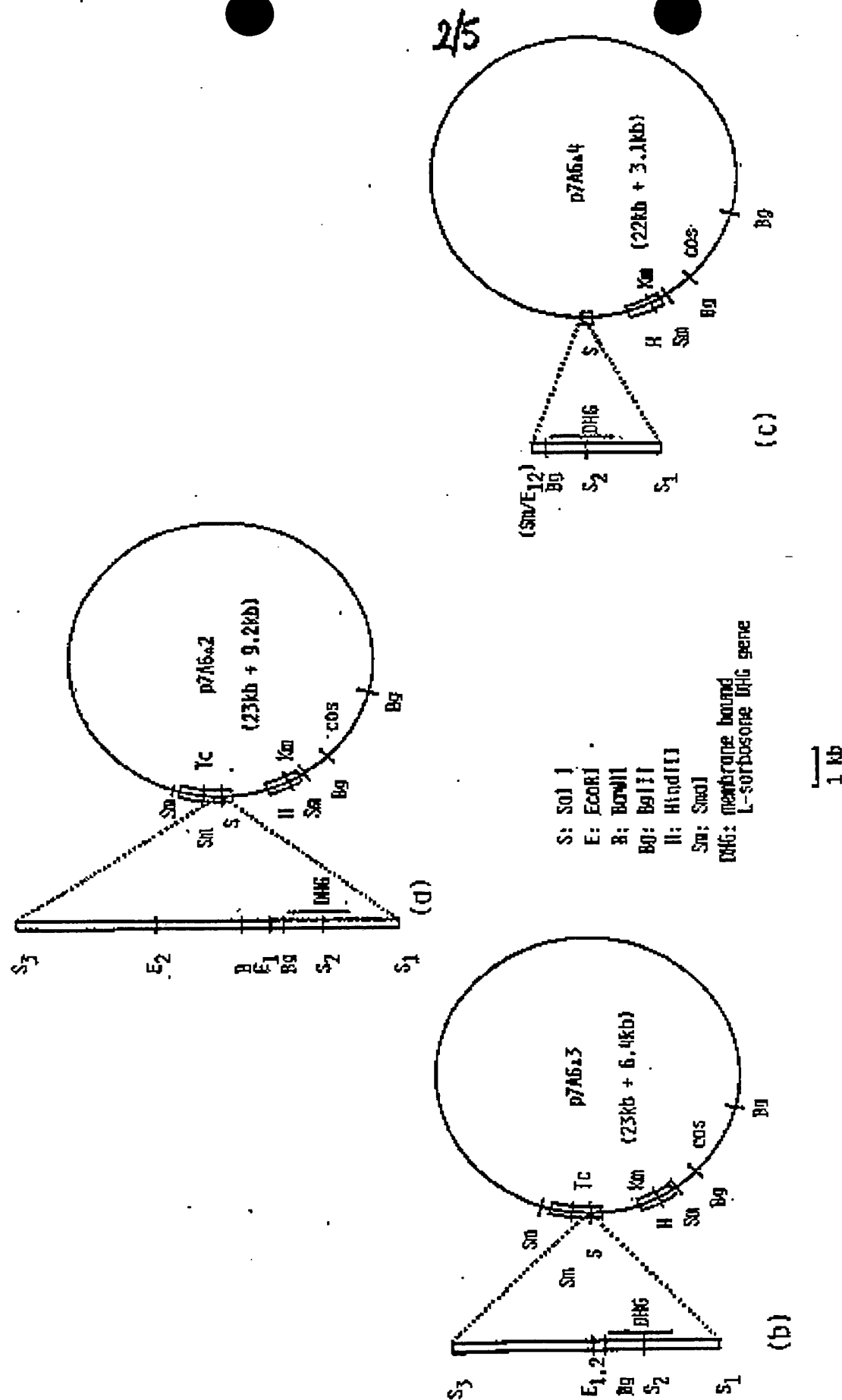


Fig. 2. Restriction map of the subclones
 (a) p7A6 Δ 2 (b) p7A6 Δ 3. (c) p7A6 Δ 4

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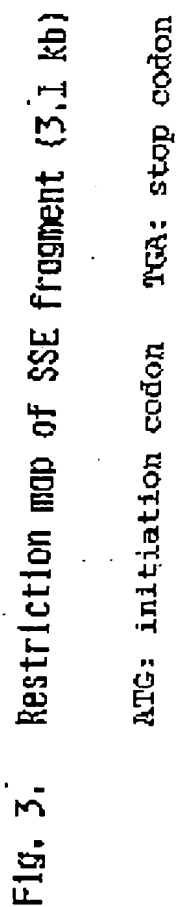


Fig. 3. Restriction map of SSE fragment (3.1 kb)

ATG: initiation codon TGA: stop codon

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1	ATG	ACC	CGT	TCC	CAG	ATC	AGG	CTT	CTC	GTC	GCG	ACC	ACC	GCC	GTC	ACC	48
1	<u>Met</u>	<u>Thr</u>	<u>Arg</u>	<u>Ser</u>	<u>Gln</u>	<u>Ile</u>	<u>Arg</u>	<u>Leu</u>	<u>Leu</u>	<u>Val</u>	<u>Ala</u>	<u>Thr</u>	<u>Thr</u>	<u>Ala</u>	<u>Val</u>	<u>Thr</u>	16
49	GCG	CTG	CTG	GTG	GCA	GCC	GCG	TAC	CGC	GCG	GTC	GTC	TCC	CCC	GAG	GAA	96
17	Ala	Leu	Leu	Val	Ala	Ala	Gly	Tyr	Arg	Ala	Val	Val	Ser	Pro	Glu	Glu	32
97	GCC	CGG	CAG	ACG	GTC	GCG	GCC	GGA	ACC	GCG	CCC	CAC	CCC	GTC	CTG	CCG	144
33	Ala	Arg	Gln	Thr	Val	Ala	Ala	Gly	Thr	Gly	Pro	His	Pro	Val	Leu	Pro	48
145	CCG	CCC	AAC	CCC	ACC	TTC	ATG	CCG	ACG	GTC	AAC	ATC	GCC	ACG	CCC	GTC	192
49	Pro	Pro	Asn	Pro	Thr	Phe	Met	Pro	Thr	Val	Asn	Ile	Ala	Thr	Pro	Val	64
193	GCG	TGG	CAG	GCG	ACG	CAG	GCC	CCG	ACC	CCG	GCG	GCG	GCG	CTG	GCG	GTC	240
65	Gly	Trp	Gln	Gly	Thr	Gln	Ala	Pro	Thr	Pro	Ala	Ala	Gly	Leu	Ala	Val	80
241	CAT	GCC	TTC	GCC	ACC	GCG	CTG	GAC	CAC	CCC	CGC	TGG	CTG	TAC	AAG	CTG	288
81	His	Ala	Phe	Ala	Thr	Gly	Leu	Asp	His	Pro	Arg	Trp	Leu	Tyr	Lys	Leu	96
289	CCC	AAC	GCG	GAT	ATC	CTG	GTG	GCG	GAA	TCC	GAG	TCC	CCC	GCG	ACC	GAC	336
97	Pro	Asn	Gly	Asp	Ile	Leu	Val	Ala	Glu	Ser	Glu	Ser	Pro	Gly	Thr	Asp	112
337	ATC	ARG	ACG	GTC	AAG	AAC	CGC	ATC	GCC	GCG	CTG	GTC	ATG	GCG	CAG	GTC	384
113	Ile	Lys	Thr	Val	Lys	Asn	Arg	Ile	Ala	Gly	Leu	Val	Met	Gly	Gln	Val	128
385	GCG	GCG	GCG	GGA	AAA	AGC	CCC	GAC	CGC	ATC	ATC	CTG	CTG	CGC	GAT	ACC	432
129	Gly	Ala	Gly	Gly	Lys	Ser	Pro	Asp	Arg	Ile	Ile	Leu	Leu	<u>Arg</u>	<u>Asp</u>	<u>Thr</u>	144
433	GAC	GCG	GAC	GCG	ATC	GCC	GAC	CAG	CGC	AGC	GTC	TTC	CTC	GAC	CAC	CTC	480
145	<u>Asp</u>	<u>Gly</u>	<u>Asp</u>	<u>Gly</u>	<u>Ile</u>	<u>Ala</u>	<u>Asp</u>	<u>Gln</u>	<u>Arg</u>	<u>Ser</u>	<u>Val</u>	<u>Phe</u>	<u>Leu</u>	<u>Asp</u>	<u>His</u>	<u>Leu</u>	160
481	TAC	TCC	CCC	TTC	GCC	ATG	GCG	CTG	GTC	GCG	GAC	ACG	CTC	TAC	GTG	GCC	528
161	Tyr	Ser	Pro	Phe	Gly	Met	Ala	Leu	Val	Gly	Asp	Thr	Leu	Tyr	Val	Ala	176
529	AAC	GCC	AAC	GCG	CTG	GTC	CGC	TTC	CCC	TAT	CAC	GAG	GCG	GAA	ACC	CAC	576
177	Asn	Ala	Asn	Ala	Leu	Val	Arg	Phe	Pro	Tyr	His	Glu	Gly	Glu	Thr	His	192
577	ATC	GAC	GCA	CCG	GCG	GAG	AAA	GCC	GTC	GAC	CTC	CCG	GCC	GCG	TAC	AAC	624
193	Ile	Asp	Ala	Pro	Gly	Glu	<u>Lys</u>	<u>Ala</u>	<u>Val</u>	<u>Asp</u>	<u>Leu</u>	<u>Pro</u>	<u>Ala</u>	<u>Gly</u>	<u>Tyr</u>	<u>Asn</u>	208
625	CAC	CAC	TCC	ACC	AAG	AAC	ATC	CTG	GCC	AGC	CCG	GAC	GCG	AGC	ACC	CTC	672
209	His	His	Trp	Thr	Lys	Asn	Ile	Leu	Ala	Ser	Pro	Asp	Gly	Ser	Thr	Leu	224
673	TAC	GTG	ACC	GTC	GCG	TCC	AAC	AGC	AAC	GTC	GCC	GAC	AAC	GCG	ATG	GAG	720
225	Tyr	Val	Thr	Val	Gly	Ser	Asn	Ser	Asn	Val	Ala	Asp	Asn	Gly	Met	Glu	240
721	GTC	GAG	GAA	GCG	CGC	GCC	CGG	ATC	GAC	CGG	TTC	GAC	ATC	GCC	ACC	GCG	768
241	Val	Glu	Glu	Gly	Arg	Ala	<u>Arg</u>	<u>Ile</u>	<u>Asp</u>	<u>Arg</u>	<u>Phe</u>	<u>Asp</u>	<u>Ile</u>	<u>Ala</u>	<u>Thr</u>	<u>Gly</u>	256
769	AAG	CTC	ACC	CCC	TAC	GCC	ACC	GCG	CTG	CGC	AAC	CCC	AAC	GAG	CTG	GCG	816
257	<u>Lys</u>	<u>Leu</u>	<u>Thr</u>	<u>Pro</u>	<u>Tyr</u>	<u>Ala</u>	<u>Thr</u>	<u>Gly</u>	<u>Leu</u>	<u>Arg</u>	<u>Asn</u>	<u>Pro</u>	<u>Asp</u>	<u>Glu</u>	<u>Leu</u>	<u>Ala</u>	272

Fig. 4

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817	TGG	GAG	CCC	AAG	ACC	GGC	GCC	CTG	TGG	GTC	GCA	GTG	AAC	GAA	CGC	GAC	864
273	<u>Trp</u>	<u>Glu</u>	<u>Pro</u>	<u>Lys</u>	<u>Thr</u>	<u>Gly</u>	<u>Ala</u>	<u>Leu</u>	<u>Trp</u>	<u>Val</u>	<u>Ala</u>	<u>Val</u>	<u>Asn</u>	<u>Glu</u>	<u>Arg</u>	<u>Asp</u>	288
865	GAA	ATC	GGC	AGC	GAC	CTG	GTG	CCC	GAC	TAC	ATC	ACG	GCG	GTG	AAG	GAG	912
299	Glu	Ile	Gly	Ser	Asp	Leu	Val	Pro	Asp	Tyr	Ile	Thr	Ala	Val	Lys	Glu	304
913	GGC	GCG	TTG	TAC	GCC	TGG	CCC	TAC	AGC	TAT	TAC	GGC	CAG	CAT	GTC	GAT	960
305	Gly	Ala	Phe	Tyr	Gly	Trp	Pro	Tyr	Ser	Tyr	Tyr	Gly	Gln	His	Val	Asp	320
961	GTC	CGC	GTC	AAG	CCG	CAG	CGG	CCC	GAC	CTG	GTG	GCC	AGC	GCC	ATC	GCC	1008
321	Val	Arg	Val	Lys	Pro	Gln	Arg	Pro	Asp	Leu	Val	Ala	Ser	Ala	Ile	Ala	336
1009	CCC	GAC	TAC	GCG	CTC	GGC	CCG	CAC	ACC	GCC	TGG	TTT	GCC	ATC	GCC	TTC	1056
337	Pro	Asp	Tyr	Ala	Leu	Gly	Pro	His	Thr	Ala	Trp	Phe	Gly	Ile	Ala	Phe	352
1057	TGG	CAG	GAC	AGC	AGC	CTG	CCC	GCG	GCC	TGG	CGC	AAT	GCG	CTG	TTC	GTC	1104
353	Ser	Gln	Asp	Ser	Ser	Leu	Pro	Ala	Ala	Trp	Arg	Asn	Gly	Leu	Phe	Val	368
1105	GCC	CAG	CAC	GGC	TCA	TGG	AAC	CGC	AAG	CCC	AAG	AGC	GCG	TAC	CGC	GTC	1152
369	Ala	Gln	His	Gly	Ser	Trp	Asn	Arg	Lys	Pro	<u>Lys</u>	<u>Ser</u>	<u>Gly</u>	<u>Tyr</u>	<u>Arg</u>	<u>Val</u>	384
1153	ATC	TAC	GTC	CCC	TTC	ACC	GAC	GTC	CAC	CCC	GAC	GGC	ACC	CCC	CGC	GAG	1200
385	<u>Ile</u>	<u>Tyr</u>	<u>Val</u>	<u>Pro</u>	<u>Phe</u>	<u>Thr</u>	<u>Asp</u>	<u>Gly</u>	<u>His</u>	<u>Pro</u>	<u>Asp</u>	<u>Gly</u>	<u>Thr</u>	<u>Pro</u>	<u>Arg</u>	<u>Asp</u>	400
1201	GTG	CTG	ACC	GGC	TTG	CTC	ACA	CAG	GAC	GAA	GAC	CAC	GCC	CAC	GCC	CGC	1248
401	Val	Leu	Thr	Gly	Phe	Leu	Thr	Gln	Asp	Glu	Asp	His	Ala	His	Gly	Arg	416
1249	CCG	GTC	GGC	CTG	GCG	CTG	GAC	AAA	TCC	GGC	GCC	CTC	CTG	GTC	GCC	GAC	1296
417	Pro	Val	Gly	Leu	Ala	Leu	Asp	Lys	Ser	Gly	Ala	Leu	Leu	Val	Ala	Asp	432
1297	GAT	GTC	GCC	AAC	ACC	CTG	TGG	GCG	GTC	ACC	GCG	ACG	GAC	CAG	AAG	ACC	1344
433	Asp	Val	Gly	Asn	Thr	Val	Trp	<u>Arg</u>	<u>Val</u>	<u>Thr</u>	<u>Gly</u>	<u>Thr</u>	<u>Asp</u>	<u>Gln</u>	<u>Lys</u>	<u>Thr</u>	448
1345	GAC																
449	Asp																

Fig. 4 DNA sequence of L-sorbose dehydrogenase of G. oxdans IF012258

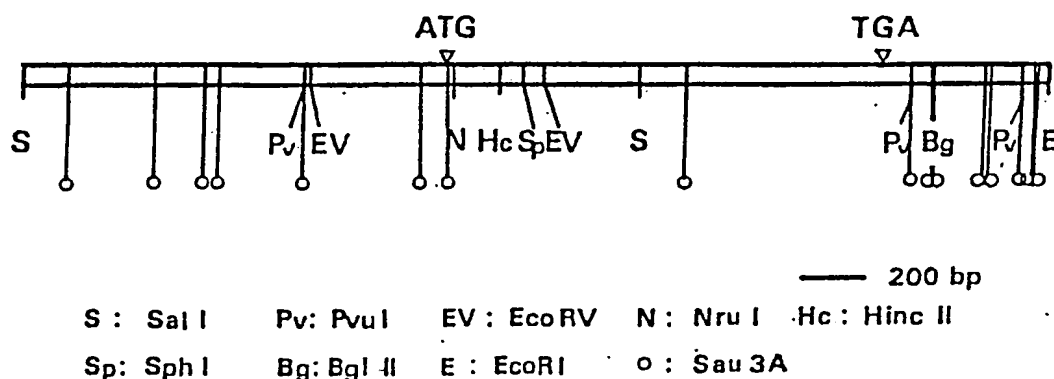
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(51) International Patent Classification 4 : C12N 9/02, 15/00, C 12 P 7/60 C 12 N 1/20		A3	(11) International Publication Number: WO 89/ 06688 (43) International Publication Date: 27 July 1989 (27.07.89)
(21) International Application Number: PCT/EP89/00010 (22) International Filing Date: 9 January 1989 (09.01.89) (31) Priority Application Number: 88100419.6 (ep) (32) Priority Date: 14 January 1988 (14.01.88) (33) Priority Countries: CH, et al.		(74) Agent: URECH, Peter; P.O. Box 3255, CH-4002 Basle (CH). (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, NL (European patent), US. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 21 September 1989 (21.09.89)	
(71) Applicant (for all designated States except US): F. HOFFMANN-LA ROCHE & CO. AKTIENGESSELLSCHAFT [CH/CH]; P.O. Box 3255, CH-4002 Basle (CH). (72) Inventors; and (75) Inventors/Applicants (for US only) : FUJIWARA, Akiko [JP/JP]; Fueta 1059-7, Kamakura-shi, Kanagawa-ken (JP). HOSHINO, Tatsuo [JP/JP]; Fueta 808-47, Kamakura-shi, Kanagawa-ken (JP). SHINJOH, Masako [JP/JP]; Dai 3 Ohfuna Park Town G-612, Kasamacho, 1555-1, Totsuka-ku, Yokohama-shi, Kanagawa-ken (JP).			

(54) Title: NOVEL ENZYME



Restriction map of SSE fragment (3.1 kb)

ATG: initiation codon TGA: stop codon

(57) Abstract

A novel coenzyme independent L-sorbose dehydrogenase originating from a microorganism belonging to the genus *Gluconobacter oxydans* which acts on L-sorbose to produce 2-keto-L-gulonic acid. The enzyme has the following physico-chemical properties: a) optimum pH: about 7.0, b) optimum temperature: about 30°C to about 40°C, c) molecular structure: consisting of one type of unit having a molecular weight of about $47,500 \pm 5,000$ as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis, d) thermostability: stable below 30°C, and e) inhibition: by Cu^{2+} ions.

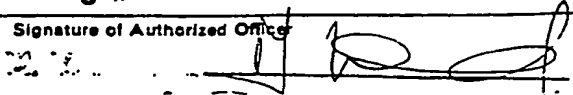
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INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 89/00010

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁴ : C 12 N 9/02, C 12 N 15/00, C 12 P 7/60, C 12 N 1/20		
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III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Biotechnology and Bioengineering, volume 17, no. 10, 1975, John Wiley & Sons, Inc., S. Makover et al.: "New mechanisms for the biosynthesis and metabolism of 2-keto-L-gulonic acid in bacteria", pages 1485-1514	1-5, 7, 9
A		6, 8, 10-18, 23-25, 30- 32, 34-36, 38-40, 42- 44, 46-48, 50
X	European Journal Applied Microbiology, volume 2, no. 1, 1975, Springer-Verlag, I. Kitamura et al.: "Metabolism of L-sorbose by enzymes from Gluconobacter melanogenus IFO 3293", pages 1-8 cited in the application	1-5, 7, 9
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Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
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International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		

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Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A		6, 8, 10- 18, 23-25, 30-32, 34- 36, 38-40
X	US, A, 3907639 (HOFFMANN-LA ROCHE INC.) 23 September 1975 cited in the application	9
P, X	EP, A, 0276832 (F. HOFFMANN-LA ROCHE & CO.) 3 August 1988 see page 11, table 3; example 3	9, 28
A	EP, A, 0248401 (F. HOFFMANN-LA ROCHE & CO.) 9 December 1987	
X	Chemical Abstracts, volume 103, no. 25, 23 December 1985, (Columbus, Ohio, US), X. Chu et al.: "Chromosome transfer in Gluconobacter oxydans mediated by pULB 113 (RP4::mini-Mu)", see page 243, abstract 207763s, & Weishengwu Xuebao 1985, 25(3), 233-8	28
X	Chemical Abstracts, volume 104, no. 1, February 1986, (Columbus, Ohio, US), see page 316, abstract 48657n, & JP, A, 60188080 (Q.P. CORP., et al.) 25 September 1985	28
X	J. Ferment. Technol., volume 63, no. 1, 1985, T. Inoue et al.: "Efficient introd- uction of vector plasmids into acetic acid bacteria", pages 1-4 see the whole article	28
X	Journal of Bacteriology, volume 145, no. 1, January 1981, Y. Murooka et al.: "Introduction of bacteriophage Mu into bacteria of various genera and intergeneric gene transfer by RP4::Mu", pages 358-368	28

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

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2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

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3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
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☒ No protest accompanied the payment of additional search fees.

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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